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Role of labellar and ovipositor taste sensilla of *Drosophila suzukii* in host recognition: a morpho-functional and behavioural approach.

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Contents

Introduction

Animals perceive their environment by the input of sensory organs, which provide crucial information about physical and chemical changes around them. Physical senses interact with diverse forms of energy such as light, sound, pressure and heat. Chemical senses, as the name suggests, detect chemicals, and the physiological response of living organisms to these stimuli is defined chemoreception. Chemical senses are a fundamental tool required for all animals to orientate themselves in the multitude of chemical cues around them. The sensory input drives a behavioural output that strongly conditions their survival and reproduction. Chemoreception consists basically of two major modalities: taste (gustation) and smell (olfaction), which are the primitive sensory systems within the animal kingdom (De Bruyne and Warr, 2006; Joseph and Carlson, 2015; Vosshall and Stocker, 2007). These two senses are classified on the basis of the physical state of the stimulus transport medium: air-borne for odorants and water-borne for tastants. Gustation is perhaps better defined as a contact sense, as the contact between the gustatory organs and the stimuli, located at a short distance, is necessarily required. By contrast, olfaction is considered as a distance-chemoreception, because stimuli, detected by the olfactory organs, come from long distances (De Bruyne and Warr, 2006; Depetris-Chauvin et al., 2015).

In particular, taste is a strategic sensory modality: in fact the discrimination ability between nutritious foods and toxic/harmful compounds is one of its main features. Therefore, the critical choice to reject or to feed on a potential food source is deeply influenced by this sense (Dethier, 1976). Moreover, in an insect life cycle, courtship, mating and oviposition are fully affected by taste (Montell, 2009).

In contrast with olfaction, which provides living organism with the ability of discriminating different odours and in different combinations, taste is a more elementary sense. In fact, in humans, and probably in most mammals, hundreds of soluble substrates are classified into only five taste qualities: sweet, bitter, sour, salty and umami (Chandrashekar et al., 2006; Lindemann, 2001; Yarmolinsky et al., 2009). Each taste quality is associated to a clearly defined behaviour. In particular, umami and sweet are palatable tastes supporting the ingestion of nutritious substances, whereas bitter and sour are unpalatable tastes promoting rejection. Salt can be attractive or repulsive, depending both on the concentration of sodium and on the physiological needs of the taster. In addition to these five taste qualities, many vertebrates and invertebrates use their gustatory system to detect other compounds such as Ca^{2+}, CO_2, water and fats (Liman et al., 2014).

Drosophila is an excellent model for studying taste perception and taste-elicited behaviours (Stocker, 2004). One of the main advantages of this

species is the extreme ease in manipulating its genome to create mutant flies, as it has been completely sequenced and a family of taste receptors has been identified (Adams et al., 2000; Clyne et al., 2000). Furthermore, gustation in *Drosophila* can be easily investigated by means of simple behavioral assays and electrophysiological investigations (De Bruyne and Warr, 2006).

Another good reason for using fruit flies as a model is that, even though their taste system has evolved quite independently from that of mammals, they show similar preferences/dislikes and detect chemicals within the same range (Amrein and Thorne, 2005; Liman et al., 2014; Yarmolinsky et al., 2009). For instance, like mice and humans, *Drosophila* is attracted to carbohydrates that provide the main food source and avoid compounds comparable to the mammalian bitters. Salts and acids are integral parts of *Drosophila* foods, and just as in mammals, those chemicals are crucial for electrolyte homeostasis (Amrein and Thorne, 2005).

The gustatory system in insects

In insect adults, contact chemoreception is mediated by hair-like structures, called chemosensilla or simply sensilla, located on the mouthparts, the legs, the wings margin and the ovipositor (Montell, 2009). Each sensillum comprises two lumina: one contains the sensory dendrites and is commonly indicated as the dendritic liquor cavity while the other, the receptor lymph

cavity, is dendrite-free and has a much larger volume (Dethier 1976; Larsen 1962). Sensory neurons are surrounded by three accessory cells (tormogen, trichogen and thecogen) (Fig. 1). These accessory cells separate the receptor lymph cavity structurally, chemically and electrically from the hemolymph space, as they are connected by septate junctions (Felt and Vande Berg, 1976; Pollack and Balakrishnan, 1997). Although taste sensilla differ in size and form, they all communicate with the surrounding environment through an apical pore, by means of which chemicals penetrate into the hair shaft. Unlike vertebrates, taste transduction is performed by bipolar nerve cells called gustatory receptor neurons (GRNs). Dendrites of GRNs interact with the taste stimuli and the axons of these neurons project directly, without synapsing, to a region of the brain called subesophageal ganglion (SOG) (Asaoka, 2002; Tang et al., 2014). The SOG does not present obvious morphological boundaries and a glomerular organization comparable to the olfactory system. In fact, taste projections are not confined to one glomerulus (Nayak and Singh, 1983; Stocker, 2004).

Normally, a typical taste sensillum houses 2-4 GRNs and 1 mechanosensory neuron with a single tubular body dendrite ending at its base (Dethier, 1976). Generally, the GRNs are described according to their sensitivity to basic stimuli (or qualities), i.e. water, sugars, salts at low or high concentration, bitter compound, etc. (Hiroi et al., 2004; Dethier, 1976; Liman et al., 2014).

In all animals, GRNs are the essential elements of taste sensory systems: they respond to gustatory stimuli by way of changes of their membrane potential, known as receptor potential, and transmit gustatory information contents to the CNS encoded as spike firing frequency. It is generally accepted that a direct correlation exists between receptor potential amplitude and spike firing frequency in both vertebrates and invertebrates (Erler and Thurm, 1981; Herness, 2000).

The gustatory system in *Drosophila*

Unlike mammals, which gustatory system is a single organ located in the head, flies sample tastants through sensilla distributed at many sites of their body surface: the proboscis, the pharynx, the legs, wings, and female genitalia (Stocker, 1994) (Fig. 2).

Drosophila species ingest food through their proboscis, which is the most important taste organ in flies. The proboscis comprises internal sensilla that line the pharynx, and external sensilla found on the labial palps, also known as labellum (Liman et al., 2014; Montell, 2009; Stocker, 1994; Vosshall and Stocker, 2007). The proboscis presents two types of sensory sensilla: taste bristles and taste pegs. Taste bristles are 31 hair-like sensilla spread on the surface of each labial palp, whereas taste pegs are about 30 shorter and simpler structures, housing only one sugar sensitive-GRN, found within the pseudotracheal grooves and exposed to stimuli only during the active

feeding (Joseph and Carlson, 2015; Montell, 2009; Stocker, 2004; Vosshall and Stocker, 2007; Yarmolinsky et al., 2009).

Other taste sensilla line the three internal sense organs: the labral sense organ (LSO), the ventral and dorsal cibarial sense organs (VCSO, DCSO) (Vosshall and Stocker, 2007). In particular, internal sensilla are thought to be involved in the final evaluation of food quality immediately after ingestion and allow the fly to make the critical decision of rejecting a potential food or to let it proceed to the digestive system (Liman et al., 2014). Both internal and external sensilla are considered to be the equivalent of the mammalian tongue.

Gustatory sensilla on the legs are associated with proboscis extension and food intake after stimulation with palatable stimuli. Some sensilla in the legs house only one bitter sensitive neuron, some only one sugar sensitive neuron and others have both GRNs (Ling et al., 2014; Meunier et al., 2003). The first leg has a different number of sensilla between males (approx. 50) and females (approx. 37). This sexual dimorphism is probably due to the presence of specialized male-specific sensilla involved in the detection of female nonvolatile pheromones, which are important for sexual behaviours such as courtship and mating (Bray and Amrein, 2003; Nayak and Singh, 1983).

The wing margin is surrounded by 40 sensilla, containing 4 GRNs each (Liman et al., 2014; Montell, 2009; Stocker, 1994; Vosshall and Stocker,

2007). These puzzling taste sensilla are related to the self-grooming behaviour in *D. melanogaster* (Yanagawa et al., 2014).

D. melanogaster females present 10 sensilla trichoidea in the genital region that may have a chemosensory function (Taylor, 1989).

Some sensilla located on the ovipositor of the blowfly *Lucilia cuprina* have been shown to have a gustatory function and are thought to have a role in mediating the search for suitable oviposition sites (Rice, 1976). Similarily, on the ovipositor of *Drosophila* there might be some chemosensory sensilla, as suggested from their morphology. However, the physiology and the molecular biology of the ovipositor sensilla is so far unknown in any *Drosophila* species (Montell, 2009; Vosshall and Stocker, 2007).

On the basis of their length and their distribution, labellar taste sensilla have been classified into three main classes: small (s-type), long (l-type) and intermediate (i-type) (Hiroi et al., 2002; Shanbhag et al., 2001) (Fig. 3). s-type and l-type sensilla contain four bipolar GRNs, whereas i-type sensilla only contains two GRNs (Amrein and Thorne, 2005; Stocker, 1994).

The GRNs fall in 4 functional classes: the S cell is activated by sugars (mono-, di- and trisaccharides), the W cell is responds to water, L1 cell is activated by low salt concentrations and the L2 cell by both high salt concentrations and bitter compounds (Charlu et al., 2013; Fujishiro et al., 1984; Hiroi et al., 2002, 2004; Rodrigues and Siddiqi, 1981; Weiss et al., 2011; Wieczorek and Wolff, 1989). The i-type sensillum lacks the W cell

and a single GRN has both L1 cell and S cell properties, while a second GRN has the L2 cell properties (Hiroi et al., 2002). A further classification has been done on the basis of the labellar sensilla responses to a panel of bitter compounds. They fall in 4 groups: i-a and i-b are intermediate sensilla norrowly tuned to complementary sets of bitter compounds; s-a and s-b are short sensilla activated by all the bitter compounds tested, but with a different pattern of activity; l-type sensilla and s-c elicit no response after stimulation with all the bitter compound tested (Weiss et al., 2011).

Neurons from taste bristles and taste pegs send their projections, via the labial nerve, to a region of the ventral brain named subesophageal ganglion (SOG): the main gustatory association center in flies (Fig. 4). The SOG does not present obvious morphological boundaries and a glomerular organization comparable to the olfactory system. Besides, taste projections are not confined to one glomerulus (Nayak and Singh, 1983; Stocker, 2004). The sensilla on the internal mouthparts send afferent fibers to the ventral part of the supraesophageal ganglion, the tritocerebrum, via the pharyngeal and accessory nerves. The wing and leg chemosensory neurons project to sensory neuropils of the thoraco-abdominal ganglion (Singh, 1997). The neurons from the thorn bristes in the *Drosophila* vaginal plate project to the most posterior neuropil of the abdominal ganglion (Stocker, 1994; Taylor, 1989).

The gustatory receptors in *Drosophila*

Gustatory receptors (GRs) were the first to be characterized as contact chemoreceptors. A family of seven transmembrane domain proteins was identified from the *Drosophila* genome database with a computer algorithm that identifies proteins on the basis of structure. Eighteen of 19 genes examined were expressed in the *Drosophila* labellum, a gustatory organ of the proboscis (Clyne et al., 2000). Subsequent analysis expanded this family to 60 GR genes, which are predicted to encode, via alternative splicing, 68 heptahelical transmembrane receptors (Robertson et al., 2003). Many of these receptors are expressed in the labellum, wings and legs (Wang et al., 2004).

GRs are distantly related to ORs, both of which have an opposite topology relative to mammalians GPCRs, with a cytoplasmatic N-terminus and an extracellular C-terminus (Benton et al., 2006; Zhang et al., 2011). Moreover, they do not share any amino acid sequence homology compared to GPCRs (Scott et al., 2001).

In flies, assays of receptor expression define segregated subsets of GRNs. For example, GRNs expressing the sweet receptor GR5a (Chyb et al., 2003) determine a diverse subset of neurons from those expressing the receptor required for bitter avoidence GR66a. Furthermore, the two populations of neurons project into nonoverlapping regions of the CNS, suggesting the

presence of a functional organization in the brain (Marella et al., 2006; Thorne et al., 2004; Wang et al., 2004).

The function of GR5a as a sugar receptor immediately suggests that this cell type (and its projections) represents a “labelled line” for sweet stimuli, while the neurons that express the GR66a correspond to a “labelled line” for bitter stimuli. In addition, behavioural, cell-ablation and imaging studies showed that all compounds that activate neurons expressing GR5a are attractive for flies, while stimuli eliting activity in neurons with GR66a are aversive (Marella et al., 2006; Thorne et al., 2004; Wang et al., 2004). So, these two pathways function as “labelled line” for sweet (GR5a) and bitter (GR66a) taste, respectively.

In mice, the activation of selective TRCs strongly controls the behaviour of the animal, regardless of the source of activation. Scott et al. (2001) found that the expression of mammalian capsaicin receptor, TRPV1, in GR5a gustatory neurons of the flies, determined a preference for capsaicin in a dose-dependent manner, while the expression in GR66a neurons produced a aversive behaviour (Marella et al., 2006). Similar experiments in which odorant receptors (ORs) were expressed in GR5a or GR66a neurons, determined attraction or repulsion to the odorants, respectively (Hiroi et al., 2008).

Finally, a recent study showed that the stimulation of GR5a neurons with blue light, after expression and activation of the rhodopsin-2 receptor, is

sufficient to start the feeding program (Gordon and Scott, 2009). Thus, just as in mammalian taste, distinct populations of GRNs are hardwired to evoke specific behavioural responses.

A recent study in flies (Gordon and Scott, 2009) identified a motor neuron in the SOG (E49) that seems to act as integrator of sweet and bitter inputs to control proboscis extension. This motor neuron is stimulated by activity in GR5a neurons and inhibited by GR66a neurons; thus, the "sweet" and "bitter" labelled lines come together to choreograph antagonistic responses in neurons that control feeding behaviour (Yarmolinsky et al., 2009).

Detection of the caloric content of sugars

One important question is whether fruit flies are able to recognize the nutritional value of sugars by only using their peripheral gustatory system. Feeding behaviour is deeply influenced by the palatability of a food source and triggered by nutritional needs, which should be satisfied by feeding on foods that ensure the animal survival. Besides, for females the ability to sense nutritious foods is crucial not only for their own survival: in fact, the female choices of a suitable oviposition site also affect the larval growth and survival.

Drosophila primarily senses sugars through tarsi and mouthparts. Tarsal contact with palatable sugars drives the extension of the proboscis, which exposes the labellar sensilla that promote, if stimulated with palatable

compounds, food acceptance and ingestion. *Drosophila* feeds on fermenting fruits rich on sucrose, glucose and fructose, all of which are palatable and nutritious foods. However, sugar sweetness is not always related to its nutritional value. On the one hand some sugars, perceived as sweets (D-arabinose), provide no nutritional-value; on the other hand some sugars (D-sorbitol) are not sensed as sweet, but support fruit flies survival (Burke and Waddell, 2011). One more example is given by synthetic sweeteners, which are appetitive, but lack nutritional value (DuBois and Prakash, 2012).

Adults of *Drosophila* can detect and remember the caloric content of different sugars, even of tasteless nutritious sugars, when associated with an odour cue (Burke and Waddell, 2011; Dus et al., 2011; Fujita and Tanimura, 2011). However, recent findings showed that mutant flies, lacking the sugar-sensitive GRs, are still able to choose calorie-rich sugars over non-nutritious sugars. These data support the idea that fruit flies evaluate the caloric content of sugars independently of taste inputs and that starvation activates unknown mechanisms that endow flies to make feeding choices based on nutritional needs rather than palatability (Dus et al., 2011).

Another study showed that *Drosophila* feeds on sugars initially in accordance with palatability. After time the preference shifts toward the caloric sugars, suggesting that taste-independent postingestive mechanisms allow animals to recognize nutritious sugars and to prefer them (Stafford et al., 2012).

Cells located both in internal organs, including the gut, and the brain express receptors that detect nutrients or their matabolically processed derivates, to regulate energy homeostatis and food behaviour. These internal sensors might detect the variations on the sugar levels in the hemolymph and trigger/inhibit the ingestion of foods (Dus et al., 2011; Fujita and Tanimura, 2011).

The fructose receptor GR43a might be an internal sensor that serves as an indicator for the consumption of nutritious sugars. The variation of sugar levels in the hemolymph supports this hypothesis. In fact, levels of trehalose and glucose, which are the main sugars in the fly's hemolymph, remain stable after feeding, wheres the fructose levels increase to a concentration that activates the fructose receptors in the brain (Miyamoto et al., 2012). Moreover, GR43a is co-expressed with GR64a in the brain nutrient sensing neurons (Fujii et al., 2015). Since GR43a does not need GR64a to function as a fructose receptor, GR64a might be another internal receptor detecting sugar level variations in the hemolymph.

Experimental model

Among the "melanogaster group" *Drosophila suzukii* Matsumara (Diptera: Drosophilidae), subgenus *Sophophora*, also known as Spotted Wing *Drosophila*, is a crop pest originated from Southeast Asia. The first outbreak,outside Asia, occurred for the first time both in North America and

Southern Europe in 2008 (Calabria et al., 2012) and currently it is continuing to spread in many areas, including Canada, America and Europe (Cini et al., 2012; Hauser, 2011). This species has the peculiarity to attack fresh and ripening fruits, unlike most other *Drosophila* species that primarily feed and lay eggs on overripe and rotten fruits. Its serrated ovipositor is an interesting feature, which allows the fly to pierce the relatively hard-skin of the fruits and to lay eggs in them. *D. suzukii* is especially fond of small fruits such as blackberries, blueberries, cherries, raspberries and strawberries (Cini et al., 2012; Lee et al., 2011; Poyet et al., 2014; Rota-Stabelli et al., 2013).

Morphology. The adult flies are small with a total lenght between 3 mm and 4 mm, a yellowish-brown thorax with black bands on the abdomen and red eyes. Males display a dark spot along the front edge of each wing and two sex combs on each tarsus. Females, as already mentioned, have a serrated ovipositor (Cini et al., 2012; Hauser, 2011).

Life cycle. *Drosophila suzukii* eggs hatch within 2 to 72 hours after being laid inside fruits, and larvae mature inside them for 3 to 13 days, until reaching the pupal stage, which lasts 3 to 15 days. During the warm season adults reach maturity one or two days after emergence, mating occurs from the first days of life and females start to lay eggs already from the second day from emergence. Females typically lay 1-3 eggs per fruit up to 16 fruits per day. Since they are able to oviposit for 10-59 days, they can lay up to a

total of 600 eggs during their lifetime (Cini et al., 2012). The duration of each stage is deeply influenced by temperature. Even if they prefer a moderate climate, adults show a high adaptation, which allows them to tolerate hot summers and withstand long periods of cold conditions and overwinter by seeking refuge under leaves, between stones, or in manmade enclosures (Calabria et al., 2012; Ometto et al., 2013; Rota-Stabelli et al., 2013; Walsh et al., 2011).

Importance for humans. *D. suzukii* is a pest threatening not only both American and European fruit industries, but also the biodiversity end the ecology of the areas where it has established, as it can alter environments and displace endemic species The major damage is caused by the larvae feeding inside the fruits. A further loss of fruits is caused by secondary infections induced by fungi, yeasts and pathogen bacteria, which can enter the fruit because of the physical damage caused by the ovipositor (Calabria et al., 2012; Cini et al., 2012; Rota-Stabelli et al., 2013).

Objectives

The role of chemoreception is vital for the animal survival. By means of chemosensory organs, animals can locate mates, suitable oviposition sites and even discriminate palatable and beneficial foods from toxic and harmful substances in their environment. *Drosophila* provides an excellent model to study chemoreception and behavioural responses deriving from the interaction with chemical stimuli. Among the melanogaster subgroup, *Drosophila suzukii* is an invasive and destructive crop pest that originated in South East Asia. Unlike most other *Drosophila* species which attack only decaying or rotten fruits, this species is extremely fond of undamaged, ripening fruits. It uses a serrated ovipositor to pierce the relatively hard-skin of fruits and lay eggs in them. This saw-like ovipositor represents one key adaptation, but other traits, such as fruit recognition mediated by the olfactory and/or gustatory systems, are also implicated. On the basis of these considerations, this work of this thesis has been divided into two separate sections.

Section I

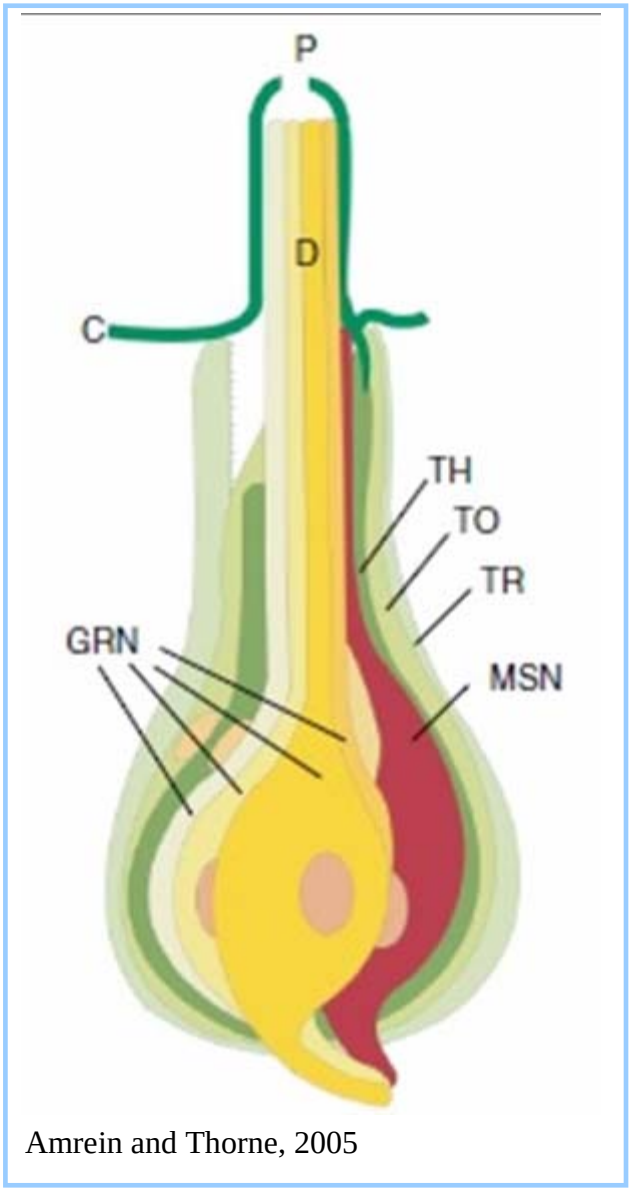
The aim of the first study was to investigate the functional significance of the sensilla housed on the ovipositor surface of *D. suzukii*, the role of which is still unknown. To determine whether these sensilla were involved in

mediating the search for suitable oviposition sites, we used a combined approach of morphological, electrophysiological and behavioural tests.

Section II

The aim of the second study was to evaluate peripheral sensitivity and palatability to different carbohydrates and assess their nutritional value, in adult insects of *D. suzukii*, by means of an electrophysiological and behavioural approach.

Figures



Amrein and Thorne, 2005

Figure 1

Scheme of a insect taste sensilla:

GRNs= gustatory receptor neurons;

MRN= mechanoreceptor neuron;

C= cuticle;

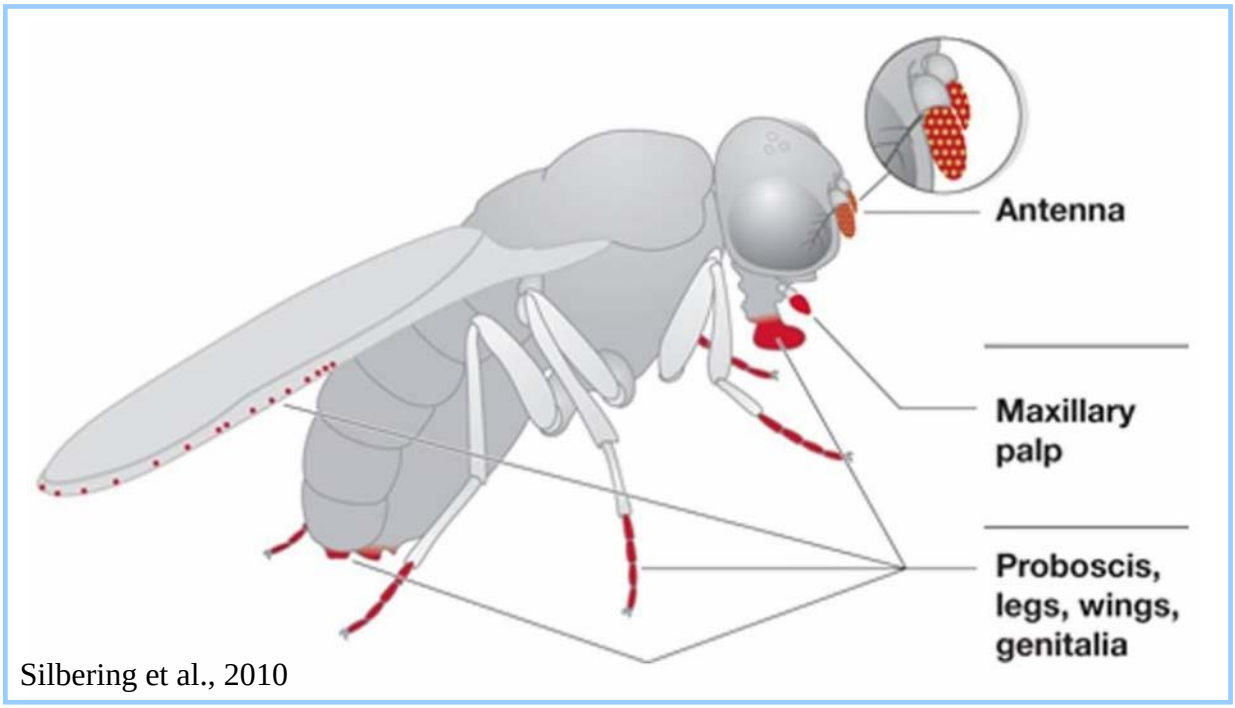
P= apical pore;

D= dendrite;

TH= thecogen cell;

TO= tormogen cell;

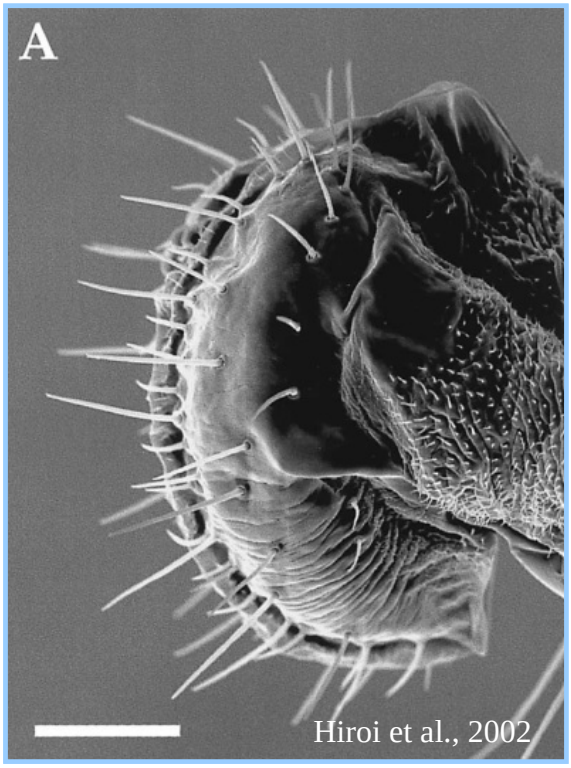
TR= trichogen cell.



Silbering et al., 2010

Figure 2

Distribution of the taste sensilla in *Drosophila melanogaster*.



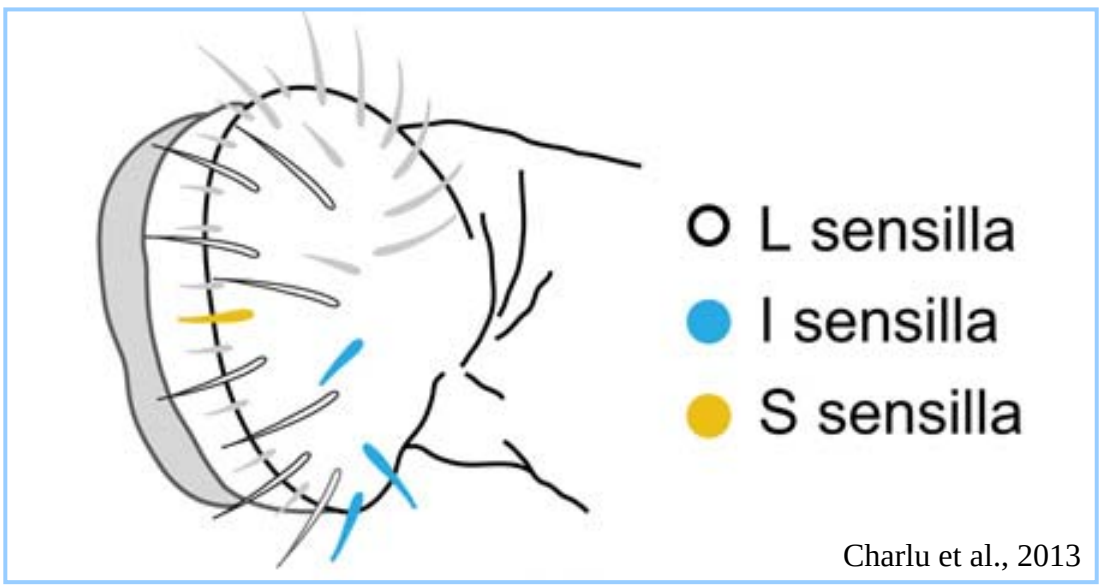
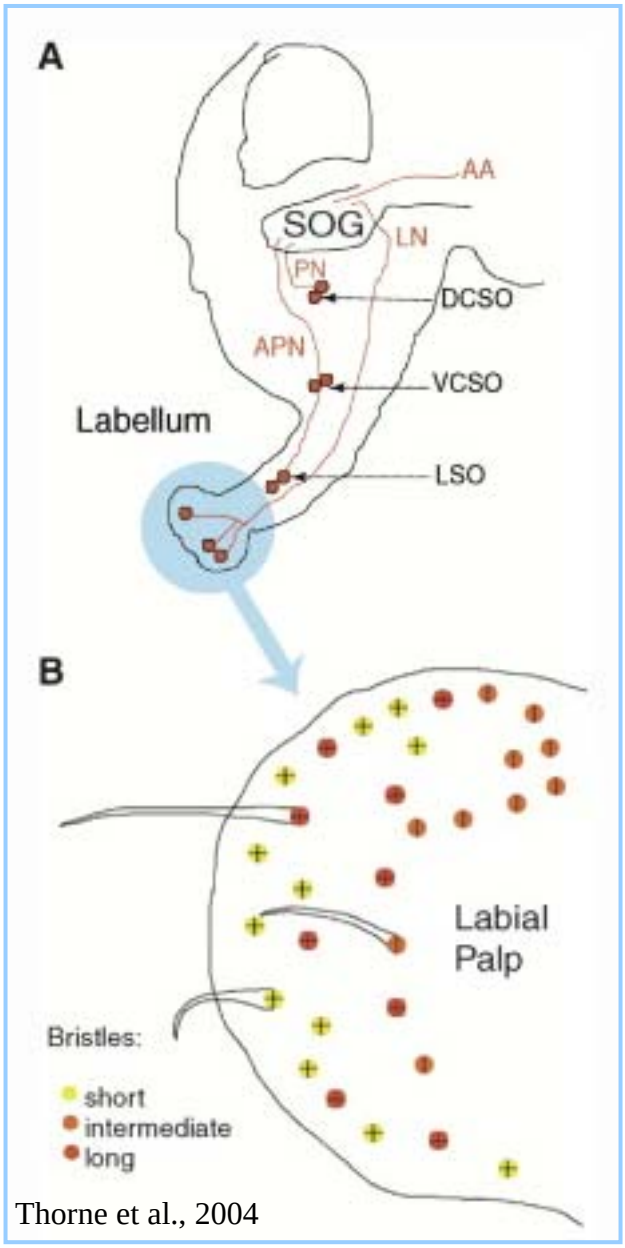


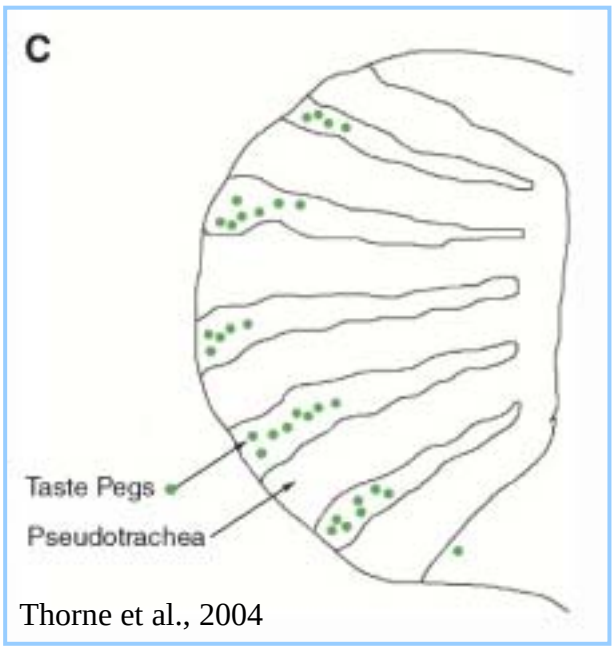
Figure 3

A. SEM image of the labellum of *D. melanogaster*;

B. Schematic representation of the distribution of short (S), intermediate (I) and long (L) labellar sensilla in *D. melanogaster*.



Thorne et al., 2004



Thorne et al., 2004

Figure 4

Neurons in taste bristles (B) and taste pegs (C) sending their projections, via the labial nerve (LN), to a region of the ventral brain named subesophageal ganglion (SOG) in *D. melanogaster.*

Morpho-functional study of the ovipositor sensilla in *Drosophila suzukii*.

Abstract

The aim of the present study was to examine the presence and the possible role of the ovipositor gustatory sensilla in *Drosophila suzukii* mediating the search for potential oviposition sites, by means of a morphological, electrophysiological and behavioural approach. The results show that the ovipositor of *D. suzukii* presents 10 single pore sensilla that respond to gustatory stimuli such as carbohydrates (sucrose, fructose and glucose), bitter compounds (nicotine and caffeine) and organic acids (ascorbic acid). Behavioural no-choice and multi-choice trials showed that the flies lay a higher number of eggs on substrates containing sugars than those with bitter or acid compounds. Our results suggest for the first time, in a *Drosophila* species, a chemosensory role for the ovipositor sensilla and their possible involvement in the choice of the oviposition sites.

Introduction

The fruit flies of the genus *Drosophila* are among the most studied insects in virtually all fields of biology given the unique combination of their reproductive (high fecundity and short generation time) and ecological (wide range of niches and fast adaptability) traits.

These features have allowed several *Drosophila* species to expand well outside their ancestral range. A classic example is *Drosophila melanogaster*, whose worldwide distribution is the result of an out-of-Africa expansion approximately 15,000 years ago (David and Capy, 1988).

Within the "melanogaster group" *D. suzukii* (Diptera: Drosophilidae) (subgenus *Sophophora*), also known as the Spotted Wing *Drosophila*, is a crop pest originated from Southeast Asia. The first outbreaks, out of Asia, occurred, both in North America and Southern Europe in 2008 and the fly currently continues to spread to new areas, including Canada, America and Europe (Cini et al., 2012). *D. suzukii* is a polyphagous insect, with a broad climate range tolerance and a high invasive potential. It became a serious crop pest everywhere it was introduced, causing heavy economic losses, and is a serious threat for agriculture. *D. suzukii* not only threatens American and European fruit industries, but also the biodiversity and the ecology of the areas where it becomes established, as it can alter ecosystems and displace endemic species. The major damage is caused by the larvae feeding on fruit pulp. A further loss of fruits is caused by secondary infections

induced by fungi, yeasts and pathogen bacteria, which can enter the fruit because of the physical damage caused by the ovipositor (Cini et al., 2012; Rota-Stabelli et al., 2013).

Unlike *D. melanogaster* that lays eggs and feeds only on decaying and rotten fruits, *D. suzukii* lays eggs on unripe and undamaged fruits (Dreves, 2011; Pham and Ryal, 2015; Rota-Stabelli et al, 2013; Walsh et al., 2011). This difference in ecology is reflected in morphological, neurological and physiological adaptations to finding unripe food sources (Ometto et al., 2013). *D. suzukii* uses a simple evolutionary advantage, a serrated ovipositor, to pierce the relatively hard skin of fruits and lay eggs in them (Rota-Stabelli et al., 2013). This saw-like structure represents one key adaptation, but as in other insects, the behavioural paradigms for host selection and localization of oviposition sites in *D. suzukii* females, as well as the search of mating partners in both sexes, are largely controlled by neural input arising from the chemical senses, olfaction and taste (Bengtsson et a., 2014; Crnjar et al., 1978; Crnjar and Prokopy, 1982; Keesey et al., 2015; Montell et al., 2009; Moon et al., 2009; Pham et al., 2015; Prokopy et al., 1982; Revadi et al., 2015; Sollai et al., 2010; Thistle et al., 2012; Xia et al., 2015).

Recognition of host-plant is mediated by the detection of odorant molecules, that are discriminated by olfactory receptor neurons (ORNs) housed within sensory structures (sensilla), located on the antennae and the maxillary

palps, and by neural input arising from gustatory receptor neurons (GRNs) housed on the labellum, tarsi and ovipositor (McBride, 2007; Ozaki et al., 2011; Roessingh et al., 1997; Ryuda et al., 2013). Understanding the relationship between the sensory input and the behavioural output in different phases of the fly life cycle is of paramount importance as it provides information useful in developing control programs employing attractants and repellents. Even though flies are highly dependent on their taste system for host location, identification of oviposition sites and search of mating partners, in the case of *D. suzukii* the knowledge concerning the tastants mediating these behaviours remains still unexplored. Any further information about chemoreception mechanisms underlying these behaviours would certainly provide power points in the tuning of novel strategies to be integrated with the biological control policies traditionally adopted (Cini et al. 2012).

D. suzukii, such as others *Drosophila* sp. (Montell et al., 2009), presents several sensilla located on the surface of its ovipositor, the functional role of which is still unknown. Ovipositor chemosensilla are known to be involved in the selection of egg-laying sites in different insect species (Sollai et al., 2010; Ryuda et al., 2013), in the present study we investigated their functional significance and their role in the choice of the oviposition sites in *D. suzukii*.

Materials & Methods

Insects

Four- to ten-day adult mated females of *Drosophila suzukii* (Diptera: Drosophilidae) were obtained from a lab-reared colony at the Dept. of Biomedical Sciences of the University of Cagliari (Italy). Flies were fed on Drosophila standard diet (Dalton et al., 2011) under controlled conditions (23°C, 70% of relative humidity, 14L/10D photoperiodic regime).

Scanning electron microscopy

Adult females of *D. suzukii* were used for the observations. Insects were anaesthetized with CO_2, and kept at -8°C until dead. Specimens were then dehydrated in a series of graded ethanol, from 50% to 99%. After dehydration, 99% ethanol was substituted with pure HMDS (Hexamethyldisilazane, Sigma Aldrich) and the specimens were dried under a hood, at room conditions. 5 specimens were mounted on each aluminum stub, taking care to place them with different orientations in order to obtain a clear view of the ventral, dorsal and both lateral sides. Mounted specimens were gold-sputtered using a Balzers Union SCD 040 unit (Balzers, Vaduz, Liechtenstein). The observations were carried out using a scanning electron microscope Philips XL 30 (FEI Company, Eindhoven, The Netherlands).

Electrophysiological experiments

Electrophysiological recordings were performed from the tip of the uniporous sensilla of the ovipositor surface, by means of the "tip-recording" technique (Fig. 1) (Hodgson et al., 1955). All recording operations were carried out by means of micromanipulators under the field of a stereomicroscope. The reference electrode, a thin Ag/AgCl, was inserted into the base of the isolated abdomen to fix the ovipositor in extended position. The recording electrode, a glass micropipette (tip diameter 20 μm), filled with the stimulating solution, was placed over the sensillum tip. All signals were recorded with a high input impedance (10^{15} Ω) electrometer (WPI, Duo 773), band-pass filtered (0.1 - 3 KHz), digitized by means of an Axon Digidata 1440A A/D acquisition system (sampling rate 10 KHz) and stored on PC for later analysis.

Taste solutions were prepared immediately before testing and were presented at room temperature. The chemical stimuli were purchased from Sigma-Aldrich, (Italy). In order to determine whether these uniporous sensilla had a gustatory function, we stimulated them with increasing concentrations of glucose and fructose (1÷1000 mM), sucrose (1÷500 mM), caffeine and nicotine (0.1÷10 mM) and ascorbic acid (0.1÷10 %). All taste stimuli were dissolved in 30 mM tricholine citrate (TCC), which was also tested alone as control (Charlu et al., 2013; Wieczorek and Wolff, 1989).

Stimuli were applied in a randomized sequence and a 3-min interval was allowed between consecutive stimulations to minimize adaptation phenomena. The 30 mM TCC (control solution) was tested at the beginning and the end of each recording sequence to assess any shift in responsiveness. To avoid drifts in solution concentration due to evaporation, a clean, dry piece of filter paper was used to draw fluid from the tip of the recording/stimulating electrode just before each recording. After each test, the ovipositor surface of the insect was rinsed with distilled water and blotted dry. Finally, we recorded only from one sensillum for fly (N=20) and no preparation was used in more than one experiment.

Data analysis

Recordings typically lasted 2-3 s, but spike analysis was performed in the interval 10 - 1010 ms after contact with the sensillum, the first 10 ms being skipped as containing the contact artifact. The first second of the discharges was chosen as representative of the phasic/phasic-tonic parts of the response (Dethier and Crnjar, 1982; Inoue et al., 2009) and spike sorting and counting were performed by means of the Clampfit 10.0 software, based on earlier studies (Dolzer et al., 2003; Dulcis and Levine, 2005; Pézier et al., 2007; Sollai et al., 2014).

Behavioural experiments

No-choice trials

Three agar solutions (8g/L) added with different tastants were prepared using sucrose 50 mM, caffeine 10 mM and ascorbic acid pH 2.5. A pure agar solution (8g/L) was used as control. When the agar solution was hardened, a square-shaped sheet of parafilm (side 4 cm) was stretched over each Petri dish, thus forming a thin layer that simulated fruit skin. Plastic containers with a fine mesh net lid were used as arenas to perform the trial. In each container only one Petri dish (sucrose or caffeine or acid) was placed together with a ball of soaked cotton as water source. Adult *D. suzukii*, 5 females and 5 males, were added to each experimental arena. Before using for the experiments, all insects were allowed to mate for 24h after eclosion. Ten replicates were carried out for each experiment. Arenas were kept in a climatic chamber (23°C; RU 70%; 14:10 L:D) and checked for ovipositions after 24 hours.

Multi-choice trials

Three agar solutions (8g/L) added with different tastants were prepared using sucrose 50 mM, caffeine 10 mM and ascorbic acid pH 2.5. A pure agar solution (8g/L) was used as control. Small petri dishes (Ø 3cm) were filled with the different agar solutions. When the agar solution was hardened, a square-shaped sheet of parafilm (side 4 cm) was stretched over

each petri dish, thus forming a thin layer that simulated fruit skin. Plastic containers with a fine mesh net lid were used as arenas to perform the experiment. In each container four petri dishes, a sweet, an acid, a bitter and a control, were placed together with a ball of soaked cotton as water source (Fig. 2). Adult *D. suzukii*, 5 females and 5 males, were added to each arena. Before being used for the experiments, all insects were allowed to mate for 24h after the eclosion. The mating arenas were kept in climatic chamber (23°C; RU 70%; 14:10 L:D) and checked for ovipositions after 24 hours. Twenty replicates were carried out for each experiment.

Statistical analysis

Repeated-measures ANOVA was used to analyze, separately for each taste stimulus (sugars, bitter compounds and ascorbic acid), the effect of increasing concentration on spike frequency.

One-way ANOVA was used to analyze the effect of the oviposition substrate (sucrose, caffeine, ascorbic acid and agar) on number of eggs laid in 24 h, for data from both no- and multi-choice condition behavioural trials. Data were checked for the assumptions of homogeneity of variance, normality and sphericity (when applicable). When the sphericity assumption was violated, a Greenhouse-Geisser correction or Huynh-Feldt correction was applied to modify the degrees of freedom. Post-hoc comparisons were conducted with the Tukey test, unless the assumption of homogeneity of

variance was violated, in which case Duncan's test was used. Statistical analyses were performed using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). P values < 0.05 were considered significant.

Results

Morphology

HRSEM images of the ovipositor of *Drosophila suzukii* are shown in Fig. 3. The ovipositor presents two valves, each housing several sensilla different in shape and length, which can be grouped in two different classes. The first class comprises a number of smooth non-porous sensilla characterized by various lengths and located at the tip of the ovipositor (green circle in fig. 3), the two longest located in the middle of the ovipositor (blue circles in fig. 3). Those of the second class, arranged in rows, show a longitudinally grooved surface; most of them are non-porous sensilla (yellow circle in fig. 3), while ten of them, 5 per valve, present an apical pore (red circles in fig. 3).

Electrophysiology

Sample of spike activity of the GRNs, recorded from the ovipositor sensilla with an apical pore, in response to different carbohydrates, bitter compounds, ascorbic acid and TCC 30 mM (solvent and control) are shown in the figures 4 and 5.

By measuring the peak-antipeak amplitude of action potentials we identified two different spike types in response to TCC alone (Fig. 6). These spikes, in the amplitude range 1÷3 mV and 3÷5 mV, were assigned to two different classes by the Clampfit software 10.0, hereafter labelled as spike-1 and spike-2, respectively. The two spike types were also present in the response to sugar solutions, bitter compounds and ascorbic acid, because TCC was added as a conducting agent to all stimuli. Two other spike types in the range 7÷10 mV (spike-3) and 13÷18 mV (spike-4) were also detected in the response to sugar solutions or bitter compounds and ascorbic acid, respectively (Figs 7-9). These results suggest the presence of four GRNs in the ovipositor sensilla and we labelled them: N1 (spike-1; 1÷3 mV), N2 (spike-2; 3÷5 mV), N3 (spike-3; 7÷10 mV in response to sugars) and N4 (spike-4; 13÷18 mV in response to bitters and ascorbic acid).

To test for a dose-response relationship, we analyzed the spike activity evoked in the first second of the discharge for each GRN to increasing concentrations of sugars, bitter compounds and ascorbic acid, by using a repeated-measures ANOVA.

Repeated-measures ANOVA (Fig. 10) showed a significant effect of concentration on the spike frequency of the N3 in response to fructose ($F[8,228]=15.458$; $p<0.0001$), glucose ($F[8,228]=8.0228$; $p<0.000001$) and sucrose ($F[8,228]=5.1255$; $p<0.0001$); post-hoc comparisons showed that the spike frequency in response to each concentration was higher than in

response to next lower concentration ($p<0.01$; Duncan's test). However, in the case of both glucose and sucrose the spike frequency increased up to 500 mM and 50 mM, respectively, indicating that the response saturation was reached. These results, together with the analysis of neural traces (Fig. 4), indicate that only one neuron (N3) is activated by sugars. Repeated-measures ANOVA (Fig. 11) also showed a significant effect of concentration on the spike frequency of N4 in response to nicotine ($F[4,252]=21.394$; $p<0.00001$), caffeine ($F[4,276]=21.264$; $p<0.000001$) and ascorbic acid ($F[4,390]=46.610$; $p<0.00001$); post-hoc comparisons showed that the neural activity in response to each concentration was higher than in response to next lower concentration ($p<0.01$; Duncan's test). These results, together with the analysis of neural traces (Fig. 5), indicate that only one neuron (N4) is activated bitter compounds and ascorbic acid and that both activate the same GRN.

Behaviour

Mean values ± s.e.m. of number of laid eggs on oviposition substrate containing agar alone or agar + sucrose 50 mM, caffeine 10 mM or ascorbic acid pH 2.5, in no-choice and multi-choice condition, are shown in figure 12. One-way ANOVA revealed, for both choice conditions, a significant interaction of the oviposition substrate with the number of laid eggs (no-choice: $F[3,36]=14.532$; $p<0.000001$ and multi-choice: $F[3,76]=6.9751$;

p=0.00033), and post-hoc comparisons showed that the number of laid eggs was significantly higher on the substrate containing sucrose than on each other substrate (no-choice: $p<0.0001$; Duncan's test and multi-choice: $p<0.05$; Tukey test). No significant differences were found in the number of eggs laid between the other substrates, for both choice conditions (no-choice: $p>0.05$; Duncan's test and multi-choice: $p>0.05$; Tukey test).

Discussion

In phytophagous insects, olfaction and taste play an important role in host selection and in the identification of oviposition sites. In Lepidopterans the oviposition behaviour can be divided in two distinct stages: flight and landing on the surface of the host, mediated by the olfactory system, followed by drumming and by bending the tail for laying eggs, mediated by the gustatory system (Ozaki et al., 2011). In fact, females determine whether a site is suitable for oviposition by drumming on the host surface and tasting the chemical compounds by means the chemosensilla located on the tarsal surface (Ryuda et al., 2013). In *Drosophila melanogaster* the choice of oviposition site appears to be a balanced decision between the information arising from both gustatory and olfactory sensory systems. In general, females are attracted to food containing acetic acid, as an oviposition substrate, but the same acetic acid promotes a positional repulsion. So, when sampling for an appropriate site, females integrate input form both sensory

modalities to choose the behavioural output between two distinct options: attraction for oviposition or positional repulsion to acid acetic. The preference to lay eggs is channeled by way of the gustatory system, while the positional repulsion through the olfactory system (Joseph et al., 2009).

In general, data in the literature showing the importance of contact chemoreception in the choice of host and oviposition site, refer to gustatory input from neurons located in the labellar and/or tarsal sensilla. In *Delia radicum* L. (Diptera: Anthomyiidae), the activation of gustatory receptor neurons of tarsal sensilla after stimulation with glucosinolates induce the oviposition behaviour (Roessingh et al., 1997); on the contrary, in *Rhagoletis pomonella* Walsh (Diptera: Tephritidae) sensory input arising from the tarsal sensilla sensitive to the oviposition deterrent pheromone (ODP) seems to be the most important stimulus inhibiting oviposition behaviour (Crnjar and Prokopy, 1982).

Conversely, very little information is available about the role of the ovipositor chemosensilla in the host choice. The primary goal of this work was to check for the presence of sensilla with gustatory function on the ovipositor surface of *D. suzukii* and then verify their involvement in the selection of the site where eggs are laid. In fact, most of the reports have studied the role of the input arising from the olfactory sensilla (Keesey et al., 2015; Pham et al., 2015; Revadi et al., 2015), while the information about gustatory input and in particular on the role of the ovipositor

chemosensilla are still unknown. We obtained SEM images showing that each valve of the ovipositor presents five sensilla arranged in a row with apical pore, suggesting a gustatory function. In agreement with the morphological results, our electrophysiological recordings indicate that these sensilla are sensitive to different tastants, confirming a role in the contact chemoreception. In fact, the dose-response relationships we found show that the activity of at least one neuron increased with increasing concentration of sugar or bitter or ascorbic acid. On the basis of the spike amplitude, the traces obtained in response to all stimuli tested suggest activity of four different neurons: two neurons activated by TCC, one by sugars and another one both by bitters and ascorbic acid. However, while we can assert with some confidence that three different spikes are present in the same neural traces in response to sugars or bitters or ascorbic acid, we cannot say with certainty whether the spikes labelled as spike-3 and spike-4 represent the bioelectric activity by one or two different neurons. The differences in amplitude may be an experimental artefact, even though it is commonly accepted that the gustatory receptors (GRs) for sweet or bitter are normally housed in different GRNs (Liman et al., 2014; Marella et al., 2006; Masek and Scott, 2010; Weiss et al., 2011). Finally, we cannot take for granted that the spike fired in response to bitter and ascorbic acid belongs to one or two distinct neurons, as in other systems acids have been shown to activate the same GRN bearing GRs for bitterness (Charlu et al., 2013).

Further morphological and functional investigations will be needed to confirm these results, since to our knowledge, only in *D. melanogaster* has been indicated the presence of about 10 gustatory sensilla on the ovipositor surface, for which the number of GRNs housed is still unknown (Montell, 2009).

The second aim of the study was to understand if the sensilla surrounding the ovipositor would provide useful information in choosing the egg-laying site. In fact, it was suggested that the spike activity of a neuron must be closely correlated with a behaviour of biological relevance for the insect. Therefore in order to understand the functional significance of the neuronal activity, the electrophysiological recordings must be coupled with the behavioural trials (Roessingh et al., 1997). In both no- and multi-choice conditions, we found that the females preferred to lay eggs on the substrate containing sucrose than on those containing caffeine, ascorbic acid or agar alone (control). Moreover, since the Petri dishes containing the substrates were covered with a thin layer of parafilm, flies could not use the inputs arising from labellum and tarsi, but only by piercing the parafilm with their ovipositor they could make contact with the substrate and detect the chemicals. These results strongly suggest that *D. suzukii* females can use the input coming from the gustatory sensilla surrounding the ovipositor to choose whether or not to lay eggs in a given substrate. It has been shown that *D. melanogaster* females probe the potential oviposition substrates first

with labellum and then with the ovipositor and that the ovipositor sensilla allow to identify sites with the preferred nutrient-conditions (e.g., sucrose) to lay their eggs (Montell, 2009; Yang et al., 2008).

In conclusion, this is the first study in *D. suzukii* showing taste sensilla in the female ovipositor and suggesting their role in the selection and choice of oviposition sites, while previous studies have highlighted the role of the olfactory system (Keesey et al., 2015; Pham et al., 2015; Revadi et al., 2015).

In addition, our findings put the basis not only to understand adaptations in behaviour, but can also be exploited to control pest insects. In fact, each year *D. suzukii* causes extensive economic damage to agricultural industries, so the identification of biologically active compounds that allow to develop alternative effective strategies to reduce fruit damage, instead of the toxic insecticides which can be dangerous to use directly on the fruits, are often the most effective and enduring ones.

Figures

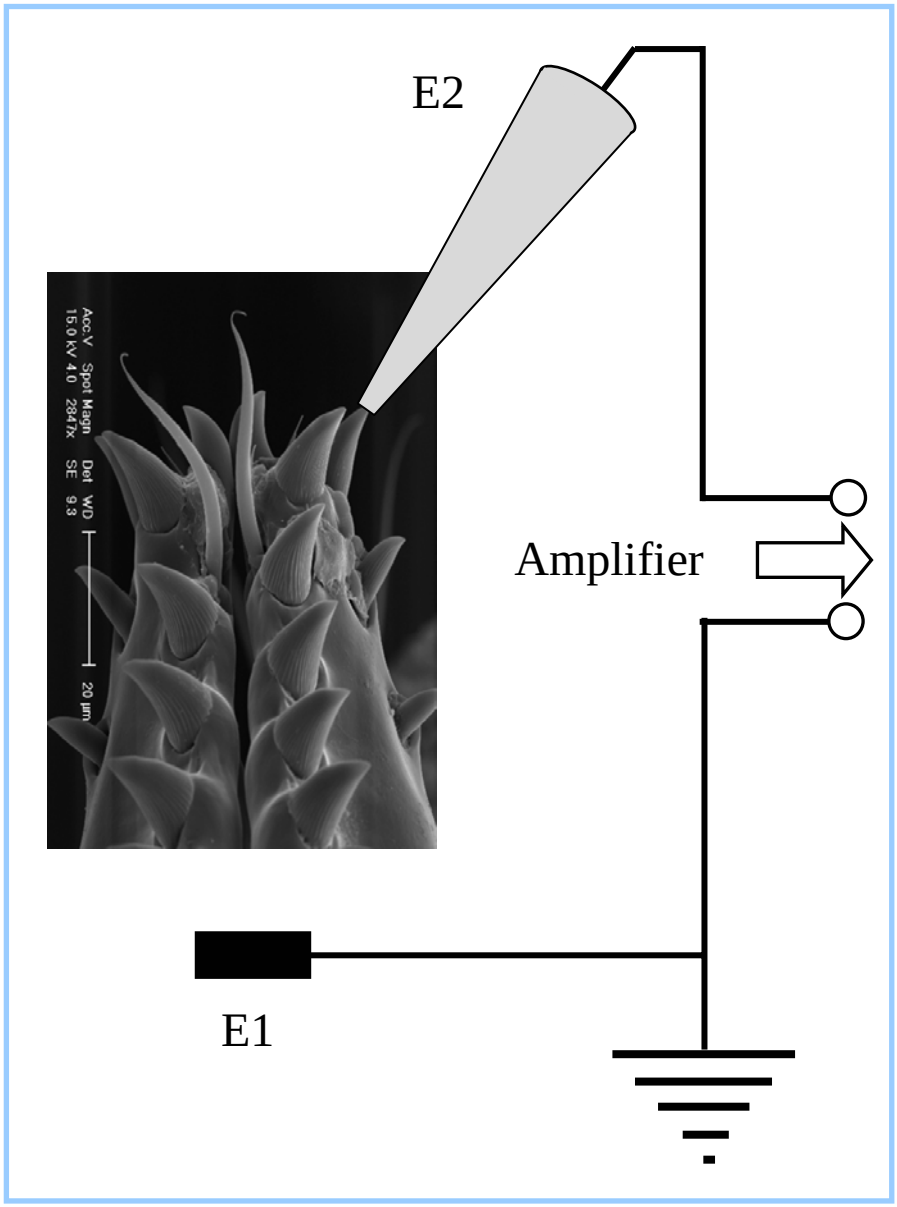


Figure 1 – Recording technique

Picture (A) and lay-out (B) showing the “tip-recording” technique:

E1= reference electrode;

E2= recording/stimulating electrode.

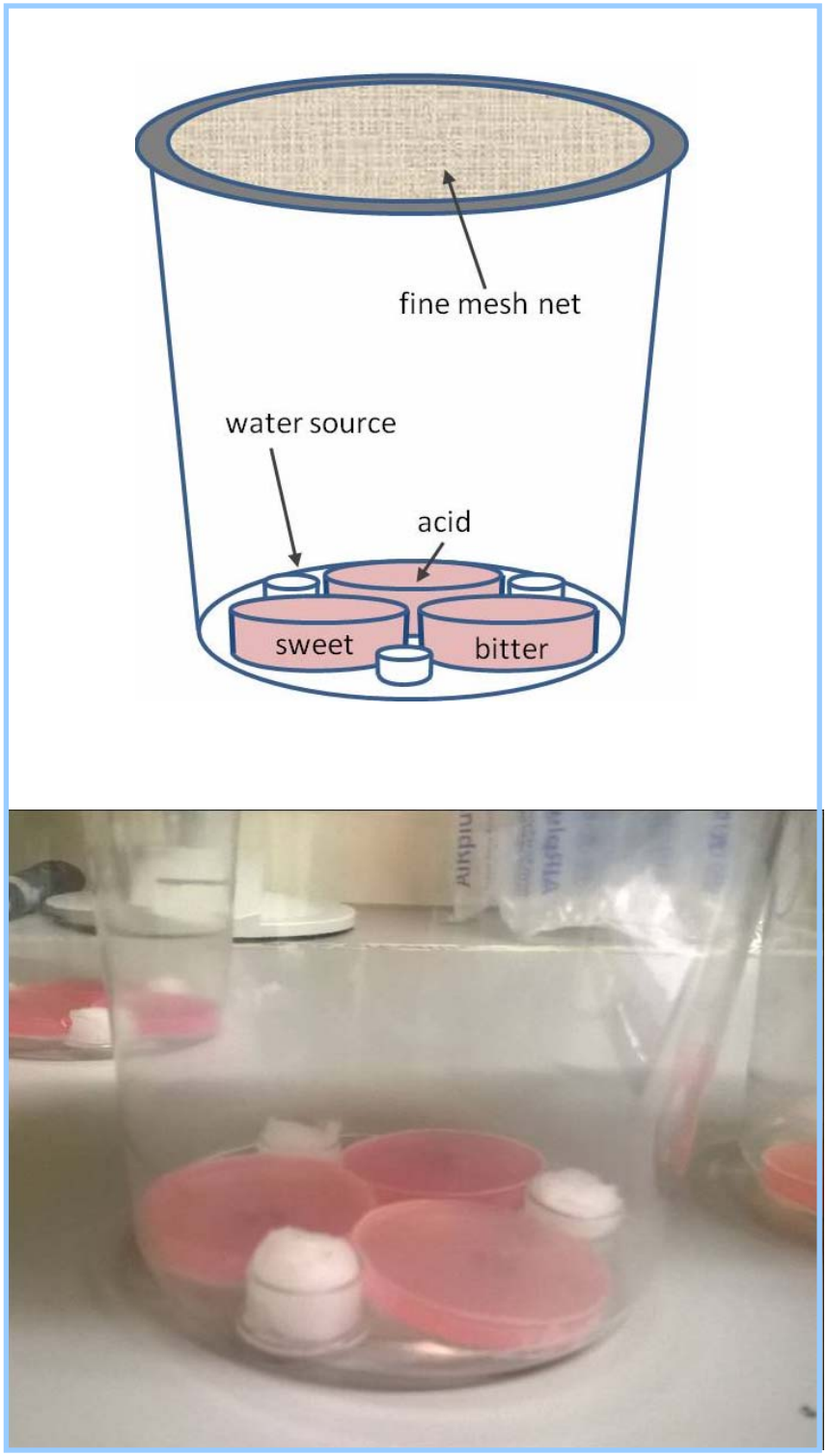


Figure 2 - Behaviour

Experimental arena for the multi-choice oviposition preference tests.

Figure 3 – Morphological results

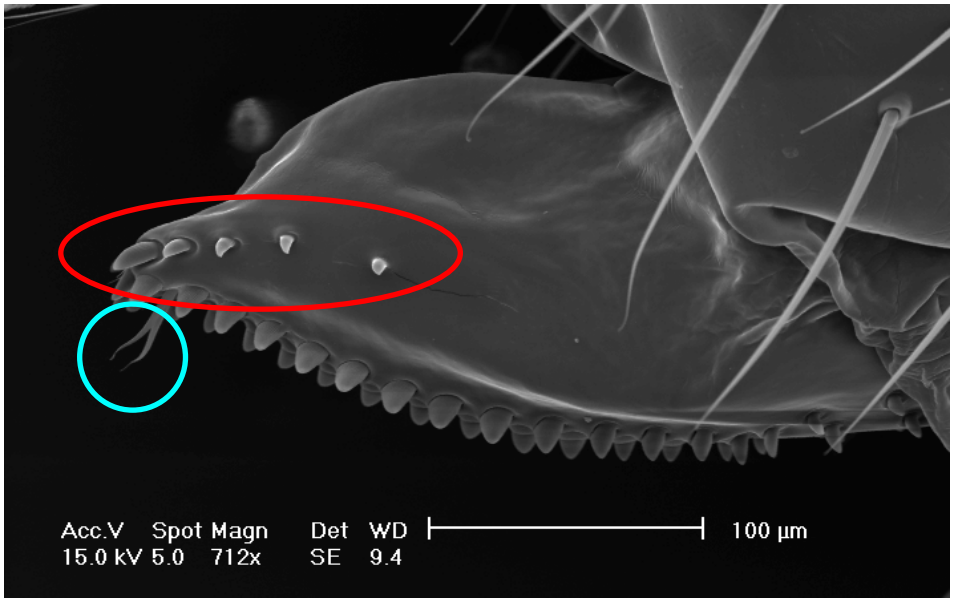
HRSEM images showing the ovipositor surface of *D. suzukii* and different types of sensilla.

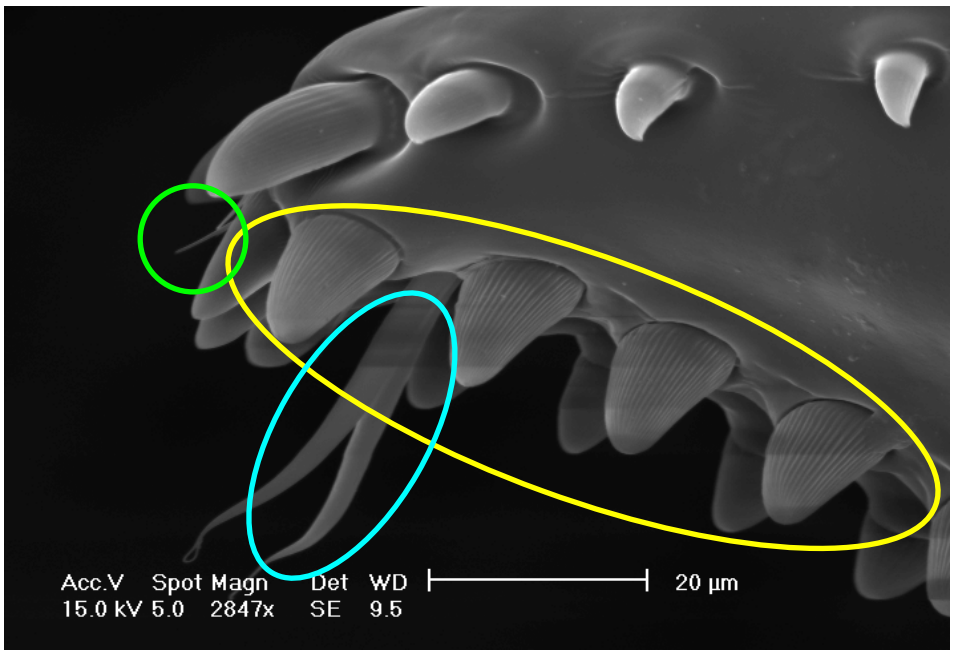
Red circles = sensilla showing a longitudinally grooved surface with an apical pore, from which the spike activity is recorded;

Yellow circle = non-porous sensilla showing a longitudinally grooved surface;

Green circle = smooth non-porous short sensilla;

Blue circles = smooth non-porous long sensilla.


Acc.V Spot Magn Det WD 100 µm
15.0 kV 5.0 712x SE 9.4


Acc.V Spot Magn Det WD 20 µm
15.0 kV 5.0 2847x SE 9.5

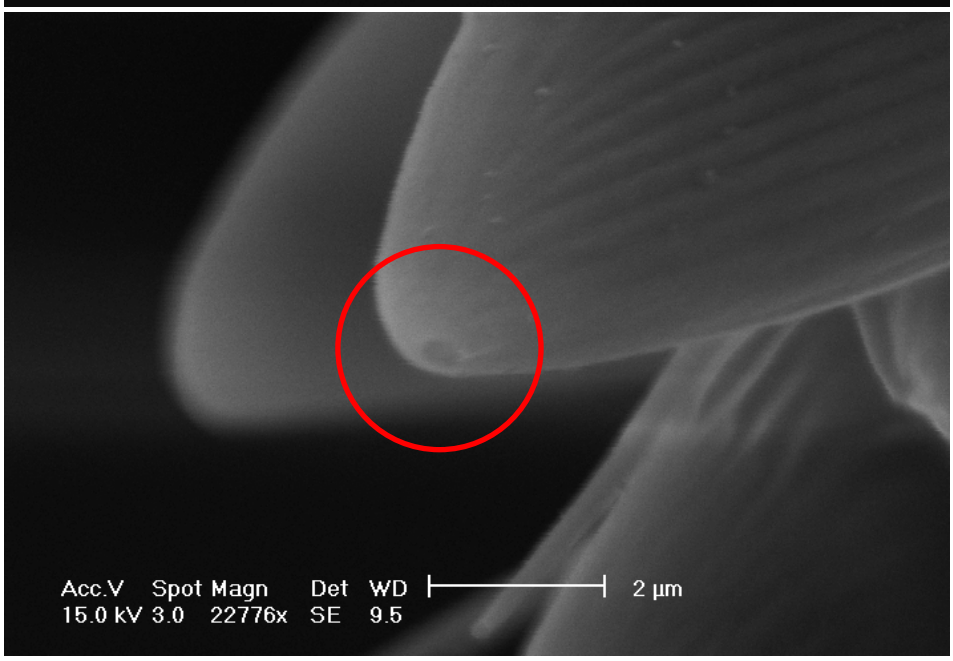

Acc.V Spot Magn Det WD 2 µm
15.0 kV 3.0 22776x SE 9.5

Figure 4 – Sample of spike discharges

Sample traces showing spike activity of an ovipositor sensillum (indicated with the red circles in figure 3) following stimulation with TCC (control), sucrose, glucose and fructose.

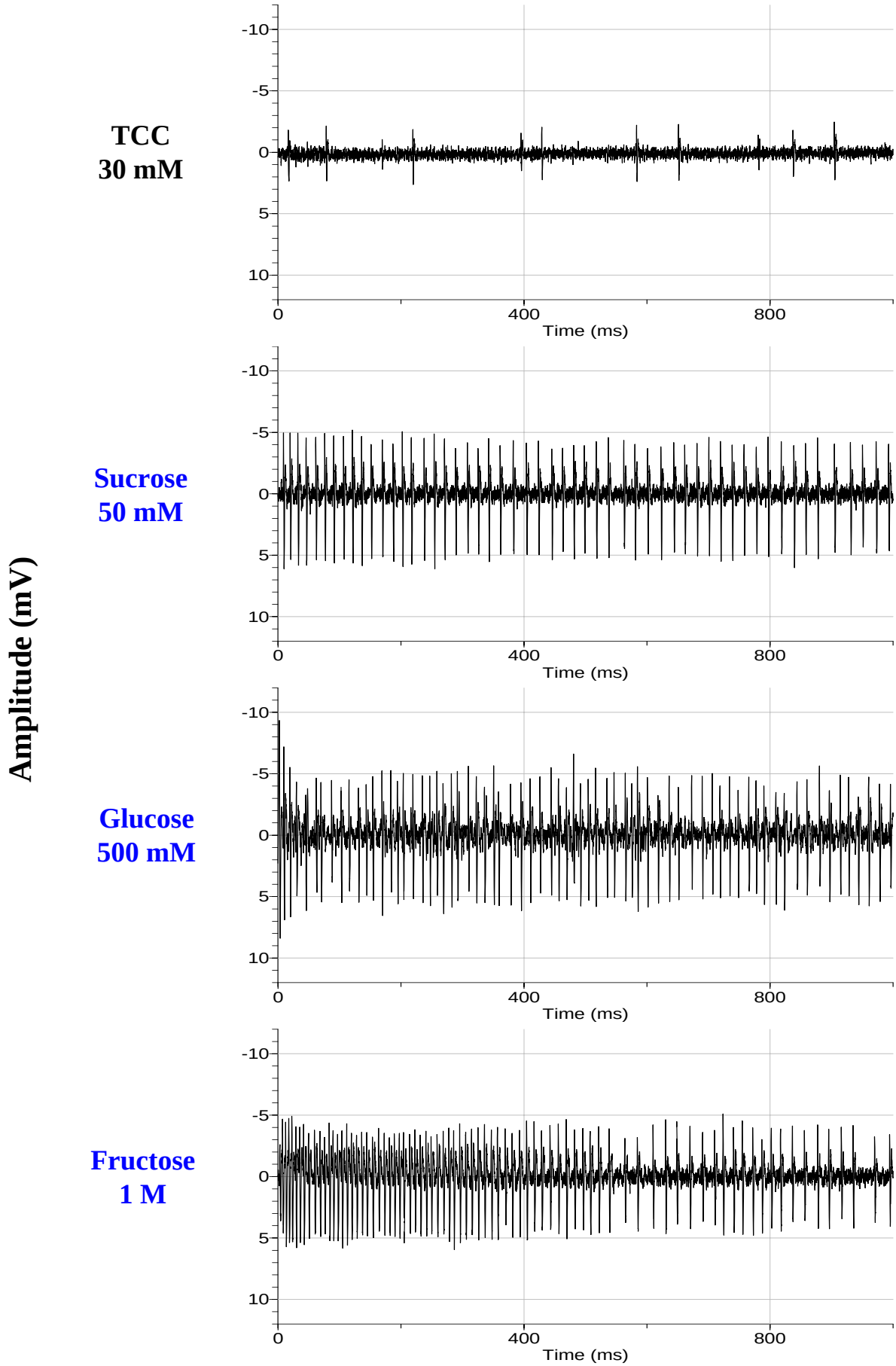
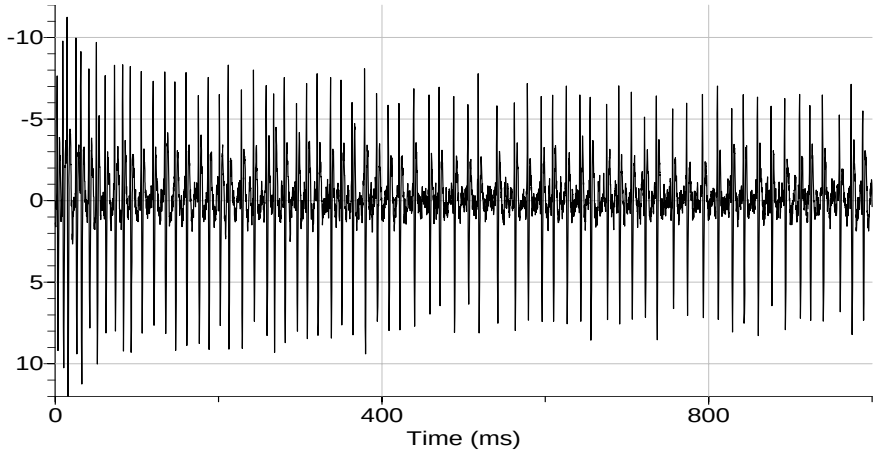

TCC
30 mM
Sucrose
50 mM
Glucose
500 mM
Fructose
1 M
Amplitude (mV)
-10
-5
0
5
10
0
400
800
Time (ms)

Figure 5 – Sample of spike discharges

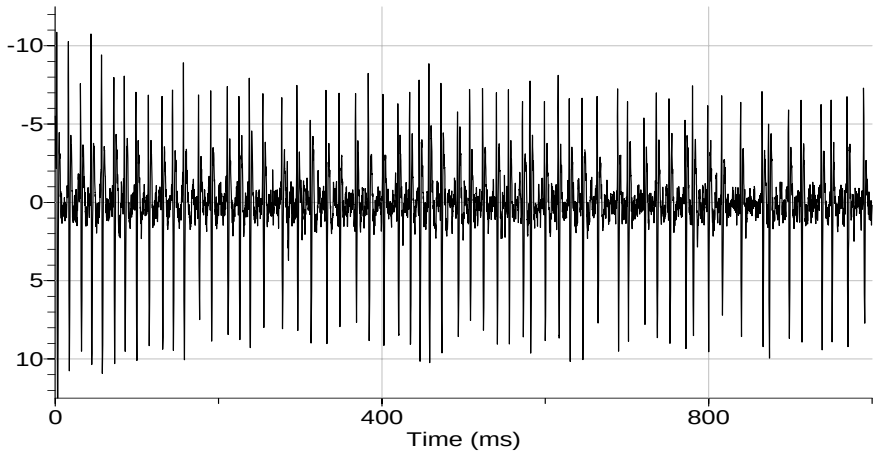
Sample traces showing spike activity of an ovipositor sensillum (indicated with the red circles in figure 3) following stimulation with nicotine, caffeine and ascorbic acid.

Amplitude (mV)

Nicotine
10 mM



Caffeine
10 mM



Ascorbic acid
10%

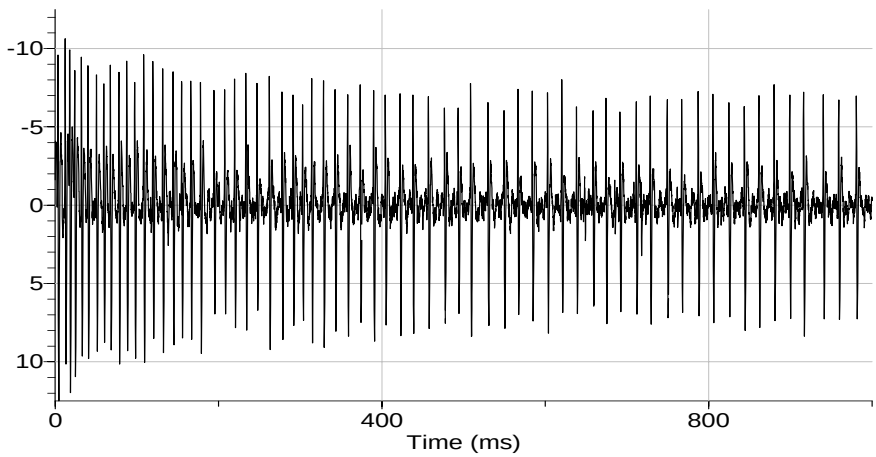
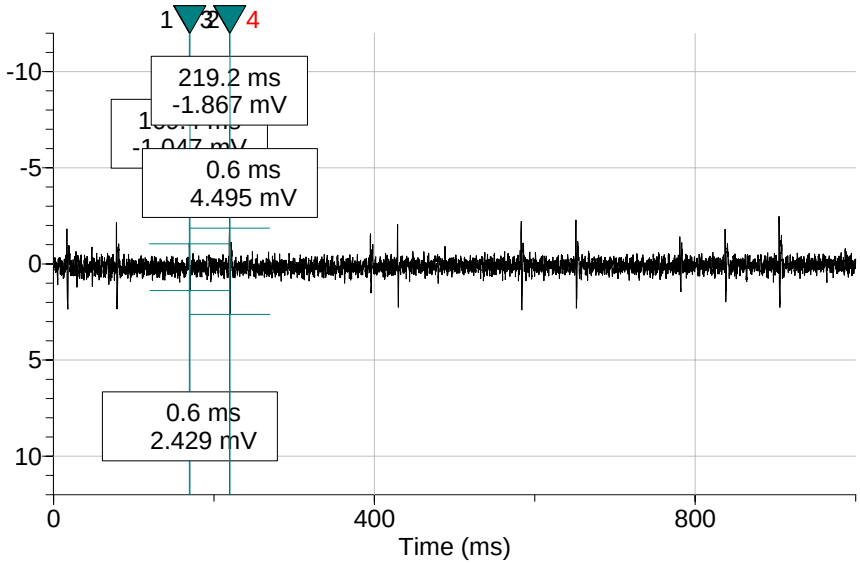
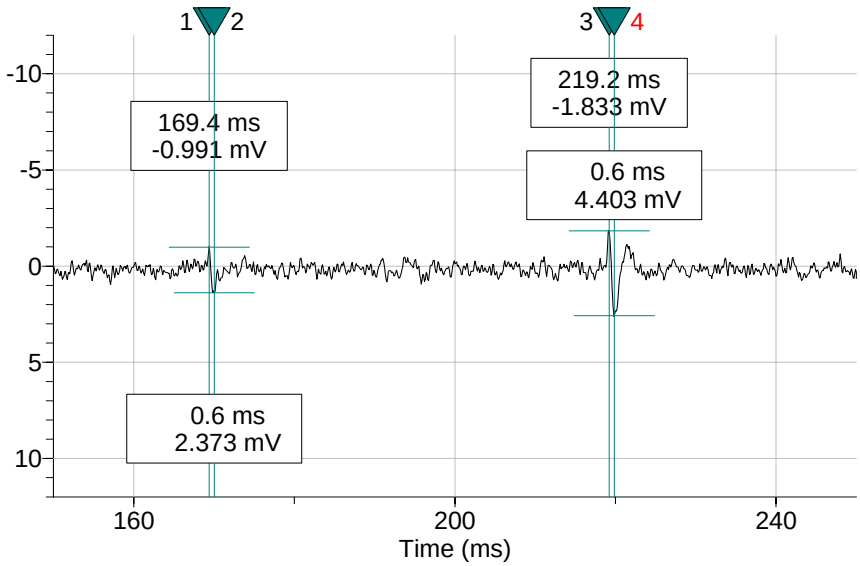


Figure 6 - Spike identification by amplitude (mV)

Spike discharge (A) and a portion thereof expanded (B) showing two different spike types, spike-1 and spike-2, in response to 30 mM TCC. Spike amplitude classes are given in the histogram (C). Vertical red dashed line is the ideal boundary of the spike types.


1
2
4
219.2 ms
-1.867 mV
0.6 ms
4.495 mV
0.6 ms
2.429 mV
-10
-5
0
5
10
0
400
800
Time (ms)


1
2
3
4
169.4 ms
-0.991 mV
219.2 ms
-1.833 mV
0.6 ms
4.403 mV
0.6 ms
2.373 mV
-10
-5
0
5
10
160
200
240
Time (ms)

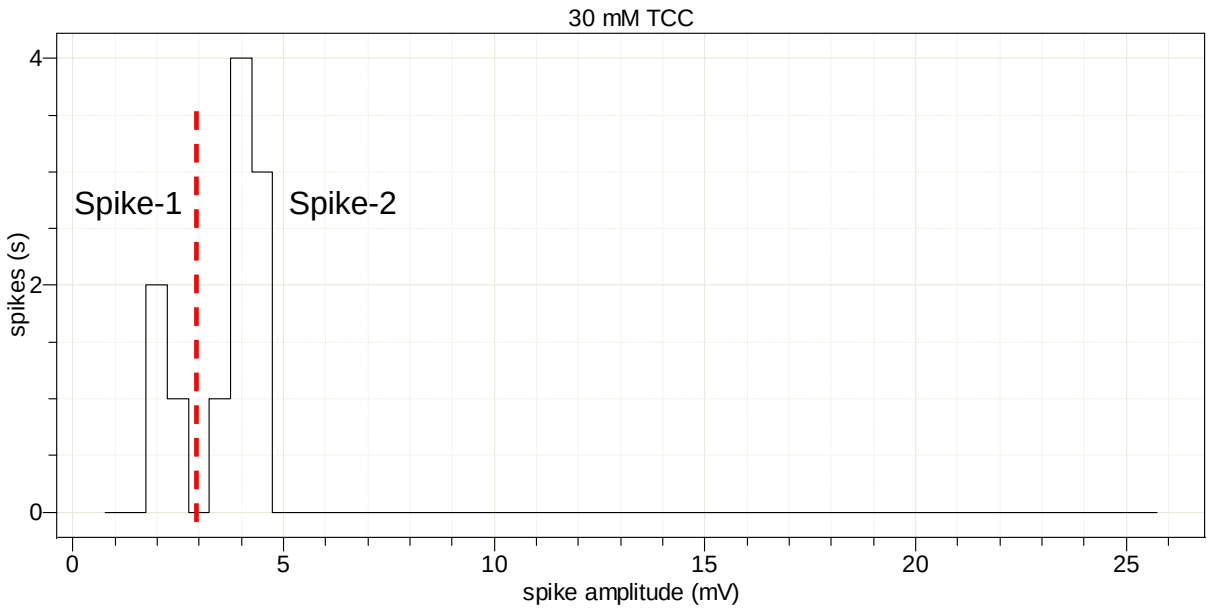
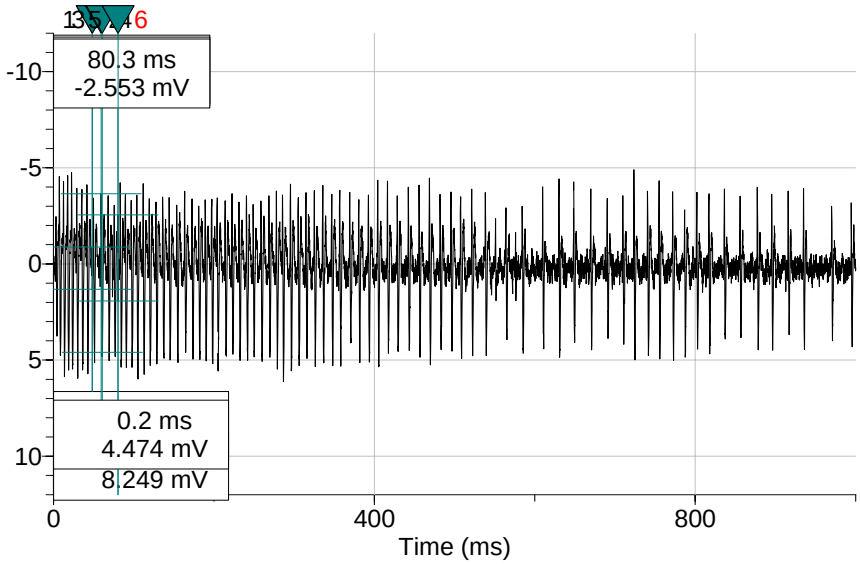
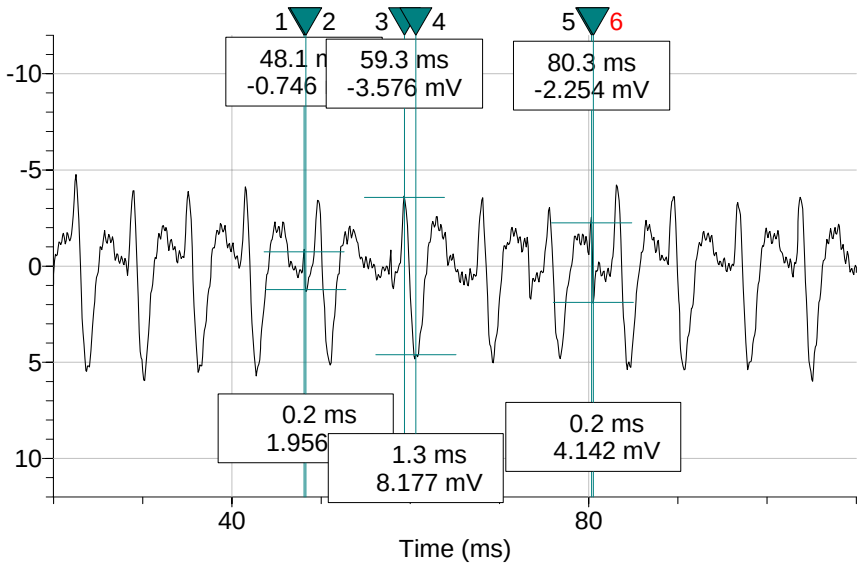

30 mM TCC
Spike-1
Spike-2
spikes (s)
0
2
4
0
5
10
15
20
25
spike amplitude (mV)

Figure 7 - Spike identification by amplitude (mV)

Spike discharge (A) and a portion thereof expanded (B) showing two different spike types, spike-1, spike-2 and spike-3, in response to 1 M fructose. Spike amplitude classes are given in the histogram (C). Vertical red dashed lines are the ideal boundaries of the spike types.


80.3 ms
-2.553 mV
0.2 ms
4.474 mV
8.249 mV
Time (ms)


59.3 ms
-3.576 mV
80.3 ms
-2.254 mV
0.2 ms
1.956
1.3 ms
8.177 mV
0.2 ms
4.142 mV
Time (ms)

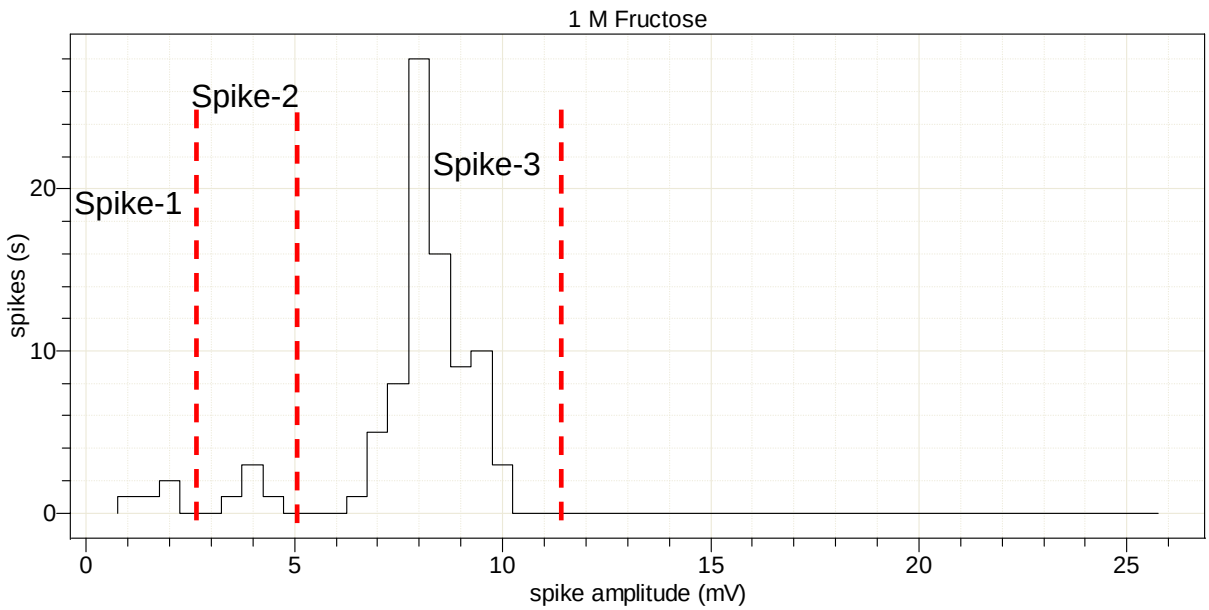
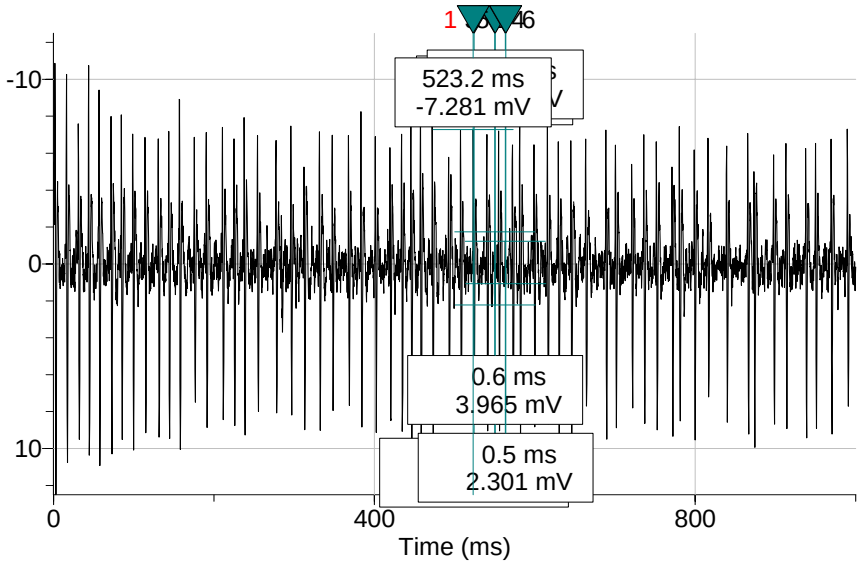
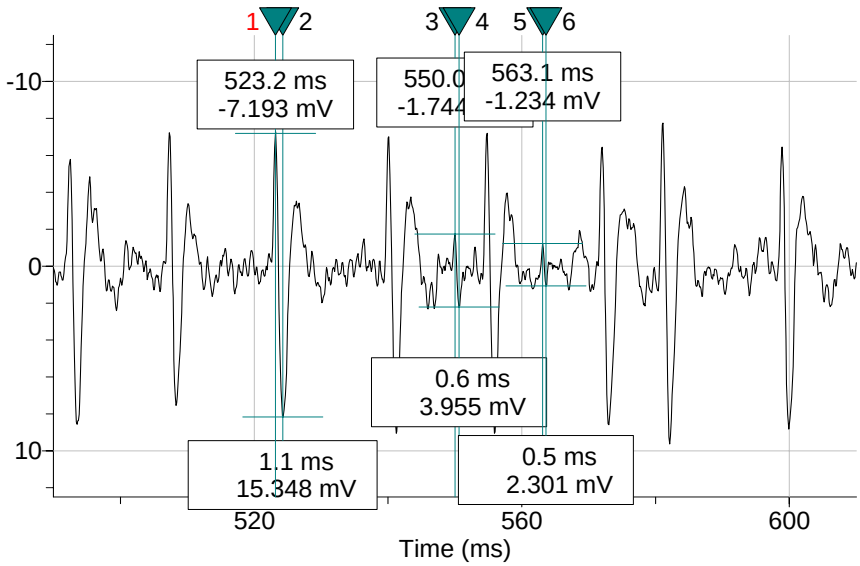

1 M Fructose
Spike-1
Spike-2
Spike-3
spikes (s)
spike amplitude (mV)

Figure 8 - Spike identification by amplitude (mV)

Spike discharge (A) and a portion thereof expanded (B) showing two different spike types, spike-1, spike-2 and spike-4, in response to 10 mM caffeine. Spike amplitude classes are given in the histogram (C). Vertical red dashed lines are the ideal boundaries of the spike types.


1
523.2 ms
-7.281 mV
0.6 ms
3.965 mV
0.5 ms
2.301 mV
-10
0
10
0
400
800
Time (ms)


1
2
3
4
5
6
523.2 ms
-7.193 mV
550.0
-1.744
563.1 ms
-1.234 mV
0.6 ms
3.955 mV
1.1 ms
15.348 mV
0.5 ms
2.301 mV
-10
0
10
520
560
600
Time (ms)

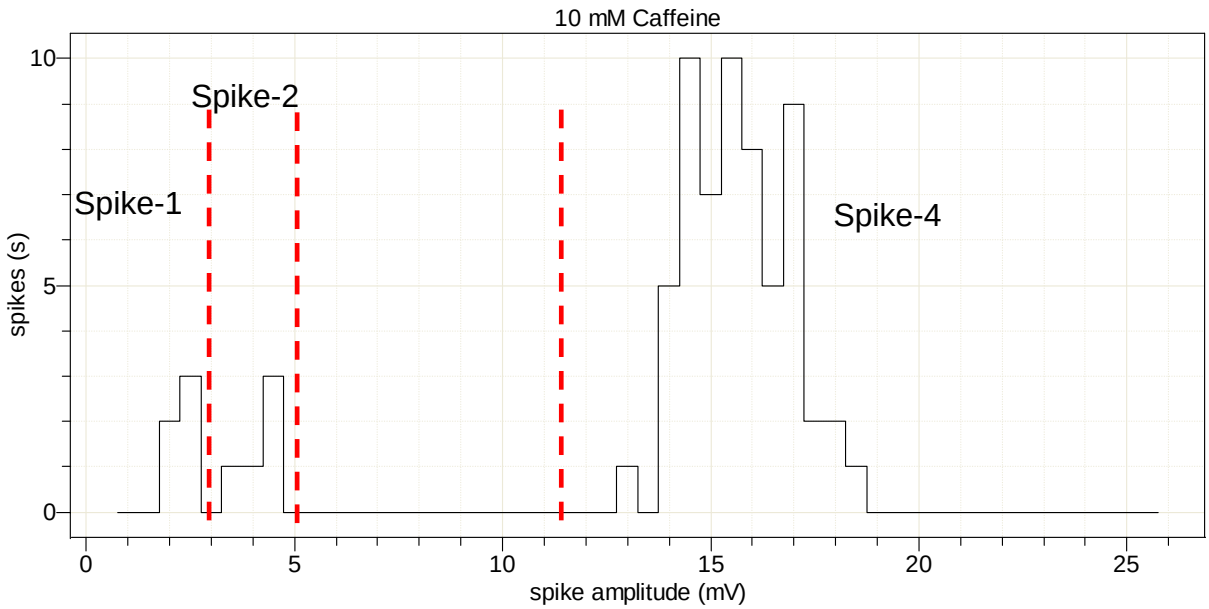
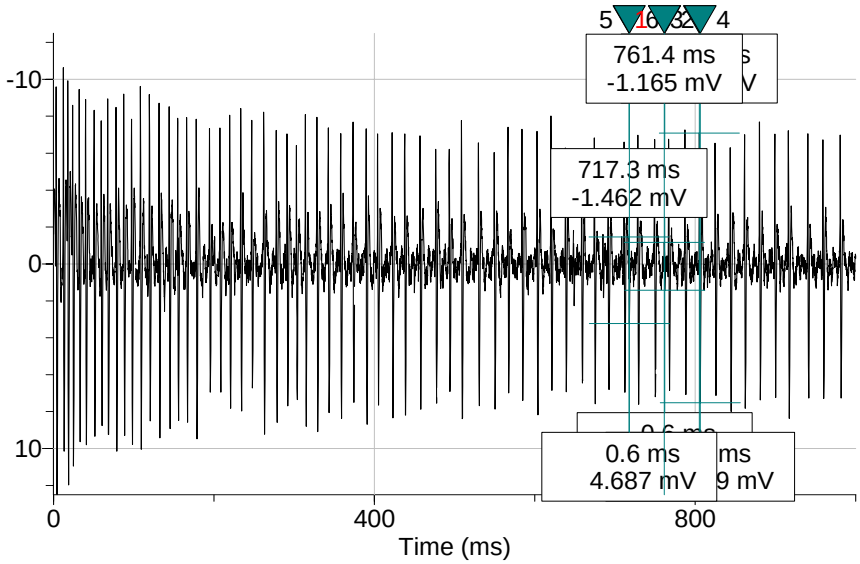
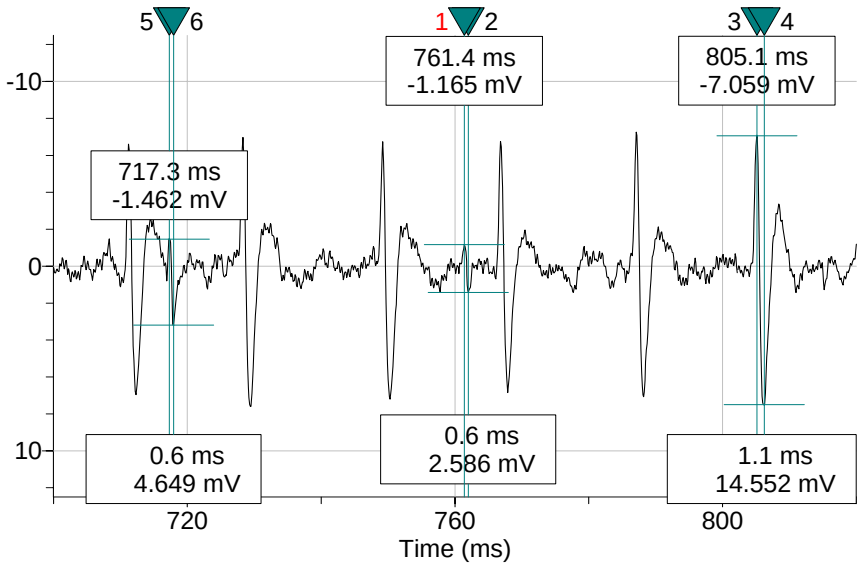
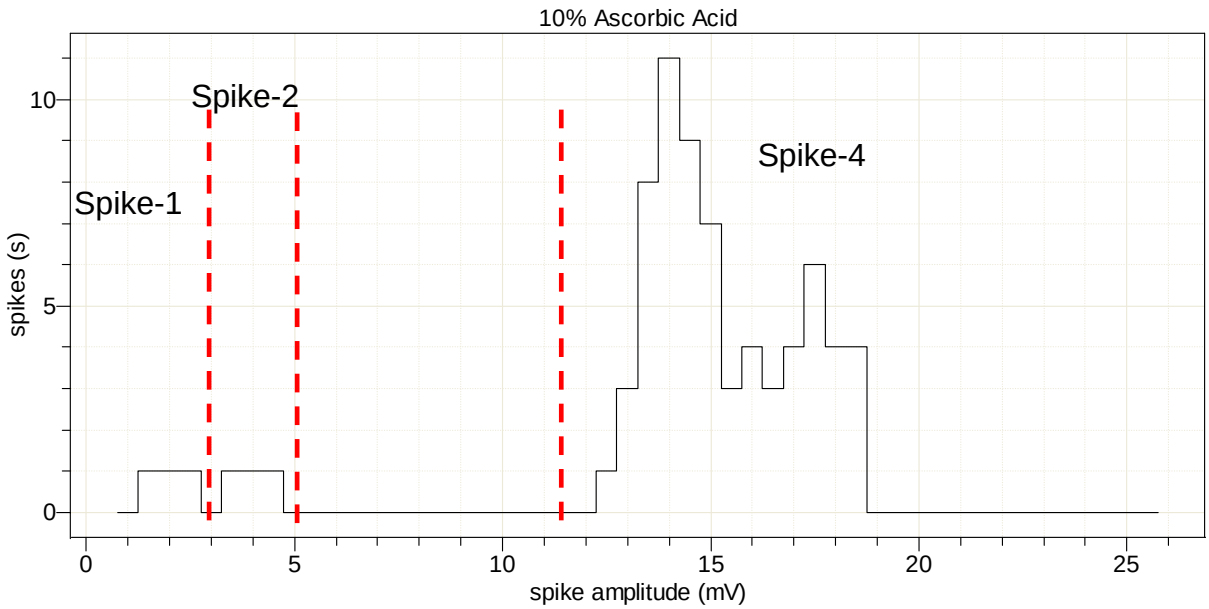

10 mM Caffeine
Spike-1
Spike-2
Spike-4
spikes (s)
10
5
0
0
5
10
15
20
25
spike amplitude (mV)

Figure 9 - Spike identification by amplitude (mV)

Spike discharge (A) and a portion thereof expanded (B) showing two different spike types, spike-1, spike-2 and spike-4, in response to 10% ascorbic acid. Spike amplitude classes are given in the histogram (C). Vertical red dashed lines are the ideal boundaries of the spike types.


761.4 ms
-1.165 mV
717.3 ms
-1.462 mV
0.6 ms
4.687 mV
-10
0
10
0
400
800
Time (ms)


5
6
1
2
3
4
761.4 ms
-1.165 mV
805.1 ms
-7.059 mV
717.3 ms
-1.462 mV
0.6 ms
4.649 mV
0.6 ms
2.586 mV
1.1 ms
14.552 mV
-10
0
10
720
760
800
Time (ms)


10% Ascorbic Acid
Spike-1
Spike-2
Spike-4
spikes (s)
0
5
10
0
5
10
15
20
25
spike amplitude (mV)

Taste sensitivity to sugars

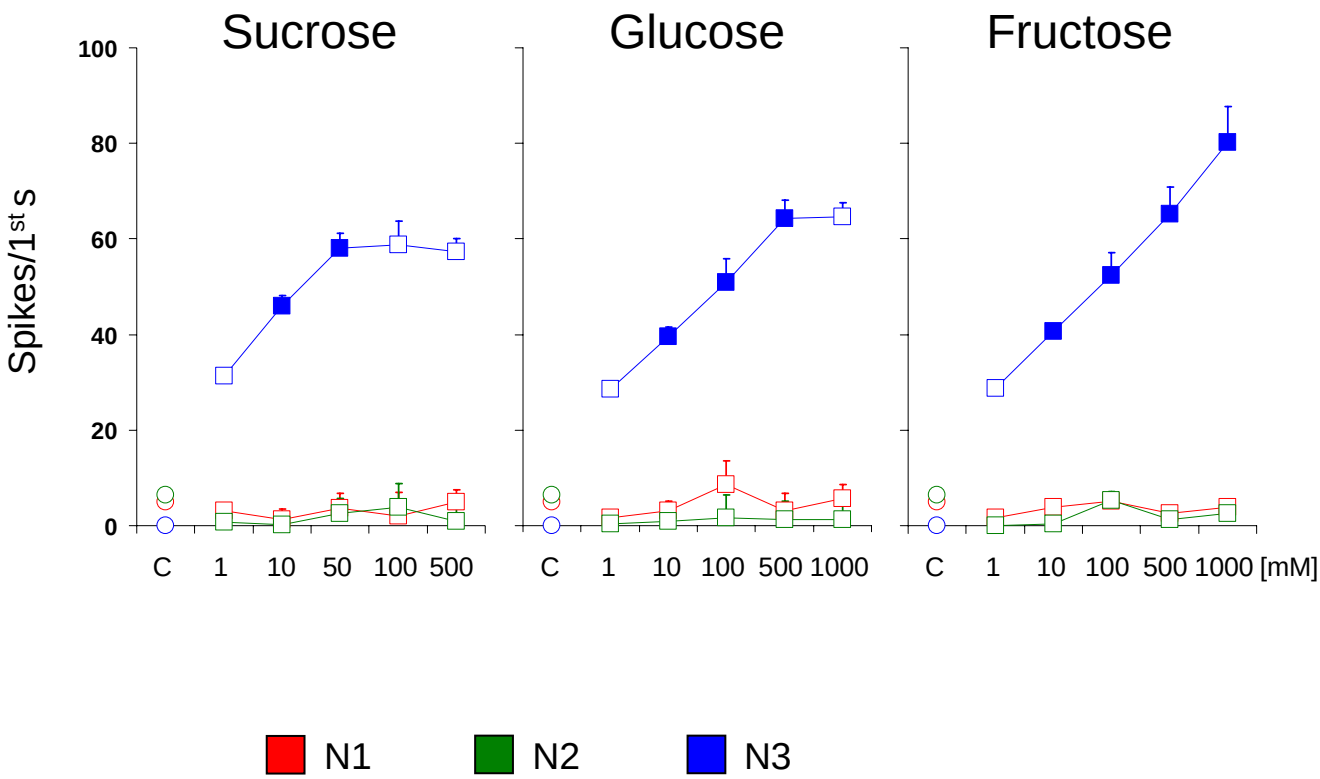


Figure 10 – Dose-response relationship

Mean values ± s.e.m. of spike frequency of N1, N2 and N3 following stimulation with increasing concentrations of sucrose, glucose and fructose. N= 20 (1 sensillum/fly). Filled symbols indicate significant differences between a concentration and that next lower ($p<0.01$; Duncan's subsequent to repeated-measures ANOVA). Circles indicate the GRN responses to 30 mM TCC (control; C).

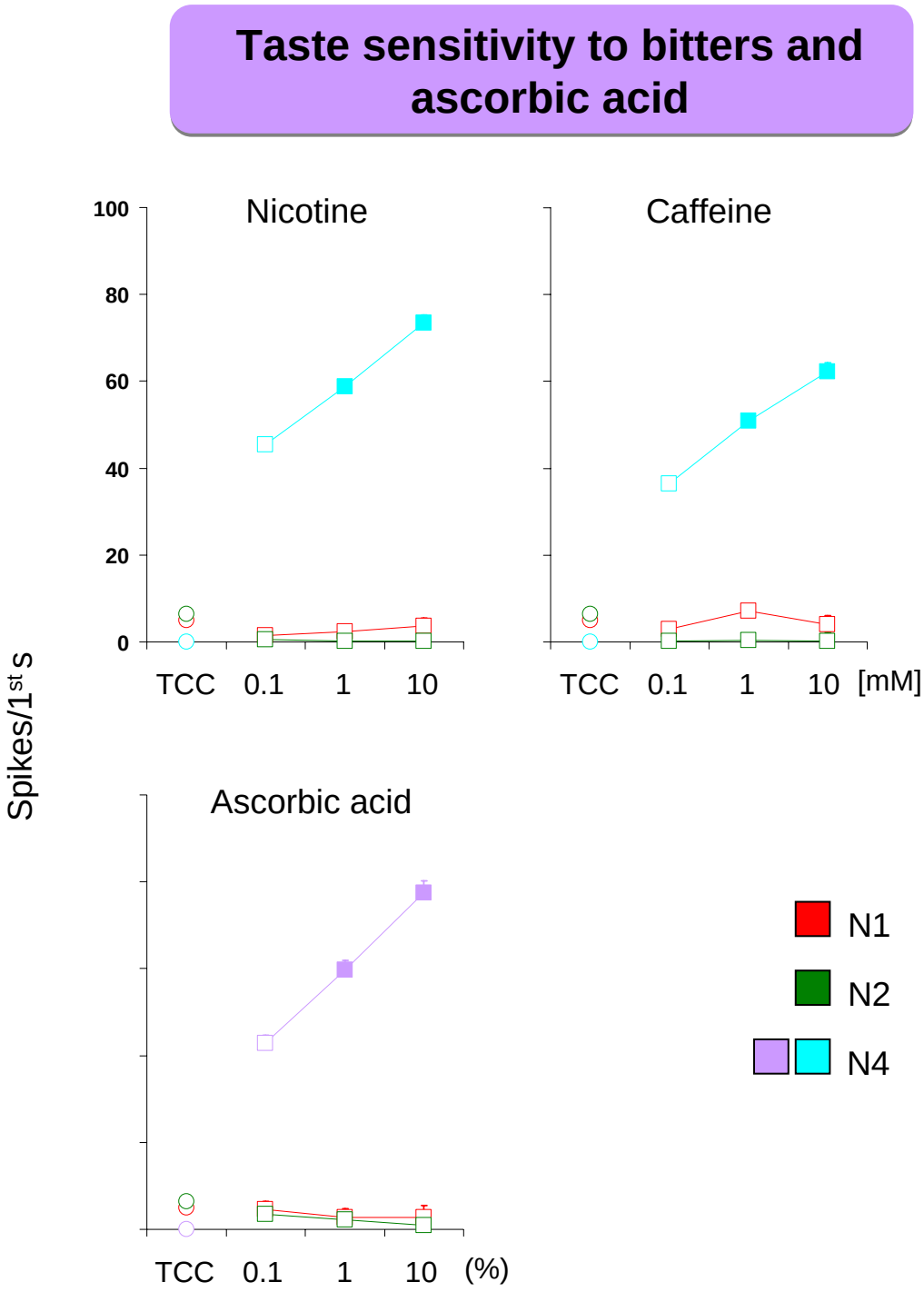


Figure 11 – Dose-response relationship

Mean values ± s.e.m. of spike frequency of N1, N2 and N4 following stimulation with increasing concentrations of nicotine, caffeine and ascorbic acid. N=20 (1 sensillum/fly). Filled symbols indicate significant differences between a concentration and that next lower ($p<0.01$; Duncan's subsequent to repeated-measures ANOVA). Circles indicate the GRN responses to 30 mM TCC (control).

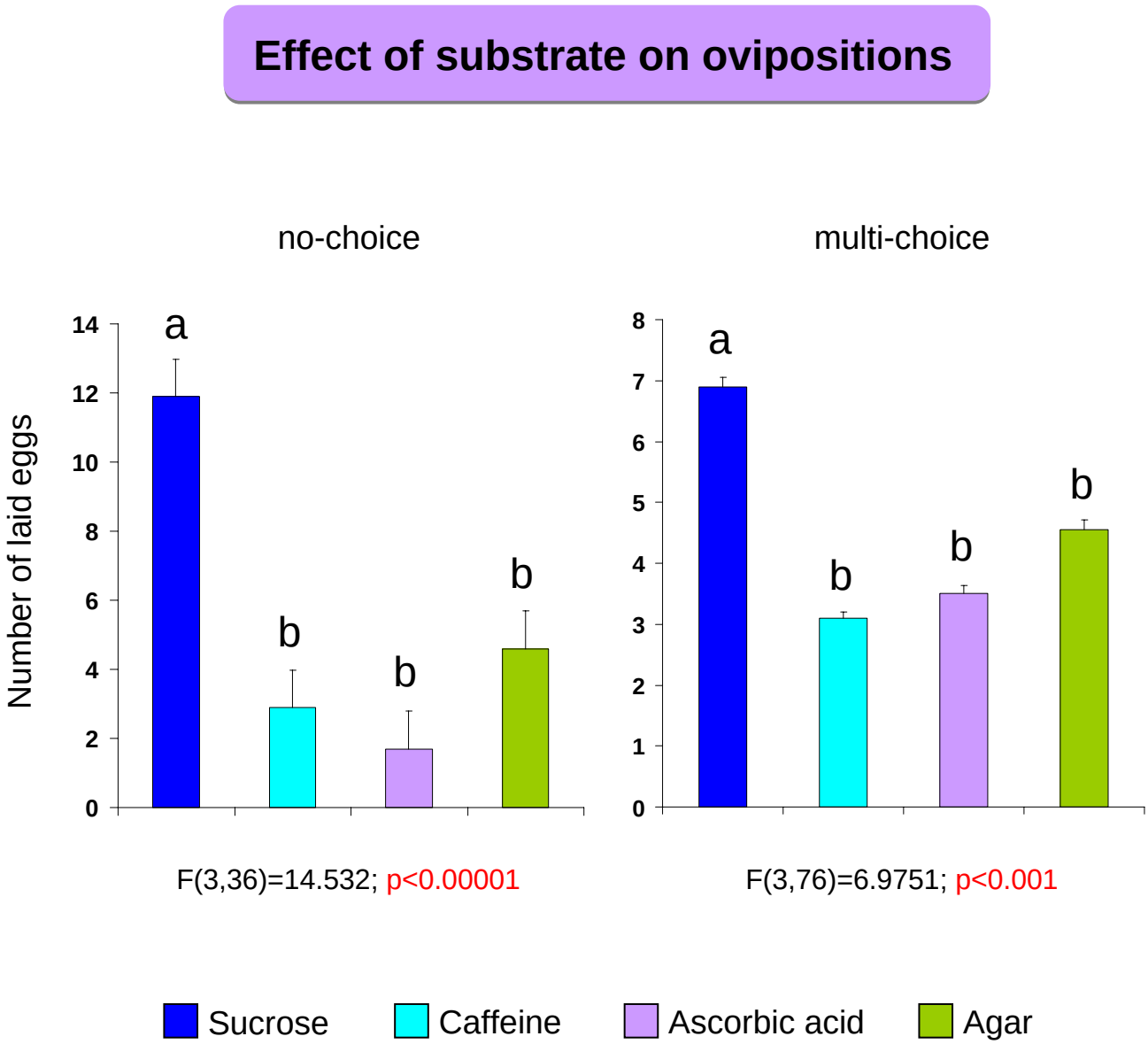


Figure 12 – Behavioural results

Mean values ± s.e.m. of number of laid eggs on oviposition substrate containing agar alone or agar + sucrose 50 mM, caffeine 10 mM or ascorbic acid pH 2.5%, in no-choice (N=10) and multi-choice condition (N=20). Different letters indicate significant differences (no-choice: $p<0.0001$; Duncan's test and multi-choice: $p<0.05$; Tukey test subsequent to one-way ANOVA).

Gustatory sensitivity and nutritional value of various carbohydrates in *Drosophila suzukii*.

Abstract

This study was aimed at evaluating peripheral sensitivity and palatability of different carbohydrates and assess their nutritional value, in adult insects of *D. suzukii* by means of an electrophysiological and behavioural approach. The spike activity was recorded from the labellar l-type sensilla stimulated with metabolizable mono- and disaccharides (maltose, sucrose, trehalose, glucose, fructose) and non-metabolizable sugars (arabinose, sucralose); the response to maltose and sucrose was stronger than to trehalose and to all monosaccharides, and that to sucralose was lower than to all other sugars. The palatability of the same sugars was evaluated by recording the proboscis extension reflex (PER). The palatability to sugars tested was: maltose = sucrose > trehalose = fructose = arabinose = glucose > sucralose. The nutritional value of the carbohydrates was assigned by means of survival trials. Flies fed on a diet containing maltose or trehalose lived longer lifespan than flies on sucrose: this suggests a higher nutritional value for the first two disaccharides. Flies fed on a diet containing sucralose or arabinose had a shorter lifespan than fructose and glucose and this suggests a lower nutritional value for the first two monosaccharides. Sugars that evoke a stronger response are also those that promote a higher activity of

PER, thus suggesting that the more stimulant is a sugar the more appetitive it is for flies. Flies fed on diets containing metabolizable sugars (maltose, sucrose, trehalose, glucose and fructose) live longer than those fed on diets with non-metabolizable sugars (arabinose and sucralose). The survival capability is longer for flies fed with disaccharides, in particular maltose, than fed with monosaccharides.

Introduction

The taste sensory system plays a central role in identifying and evaluating potential foods by discriminating between nutritious chemicals that promote feeding, and structurally diverse, harmful, or even toxic compounds, that inhibit feeding (Miyamoto et al., 2012). Therefore, the critical choice to reject or to feed on a potential food source is deeply influenced by this sense (Dethier, 1976).

Insects offer several advantages for the study of peripheral taste sensitivity. Unlike the case of vertebrates, taste transduction is performed by bipolar neurons called gustatory receptor neurons (GRNs), housed within bristles called taste sensilla, located on the labellum, legs, wing margins and ovipositor (Jiao et al., 2007; Yarmolinsky et al., 2009). Taste sensilla have an apical pore that enables to record the neural activity originating from single GRNs (Hodgson et al., 1955). In *Drosophila melanogaster* labellar taste sensilla have been classified on the basis of their length and their distribution, into three main classes: small (s-type), long (l-type) and intermediate (i-type (Hiroi et al., 2002; Shanbhag et al., 2001). The s-type and l-type sensilla contain four bipolar GRNs, whereas i-type sensilla bear only two GRNs (Amrein and Thorne, 2005; Stocker, 1994).

The GRNs fall in 4 functional classes: the S cell is activated by sugars (mono-, di- and trisaccharides), the W cell is tuned to water and L1 and L2 cells are activated by low salt and high salt, respectively (Fujishiro et al.,

1984; Hiroi et al., 2002; Rodrigues and Siddiqi, 1981; Wieczorek and Wolff, 1989). The i-type sensillum lacks the W cell and a single GRN has both L1 cell and S cell properties, while a second GRN has L2 cell properties (Hiroi et al., 2002). The L2 cell in i-type and s-type sensilla is activated not only by high salt but also by bitter compounds and low pH solutions (Charlu et al., 2013; Hiroi et al., 2004; Weiss et al., 2011).

The stimulation of labial or tarsal taste neurons with an attractive stimulus such as a sugar determines the proboscis extension, the spreading of the labella and the start of feeding. On the contrary, the addition of a unpleasant compound to the food source suppresses the proboscis extension reflex (PER) and elicits retraction.

An important feature of the neural circuits that control feeding is that they integrate information about palatability (taste) and nutritional content of the food source. These variables are often related to each other; in fact, sugars generally represent a good source of carbohydrates and have a sweet taste palatable. However, the intensity of the sensory response to a specific sugar is not always indicative of its nutritional value, and recent data suggest that insects can detect the caloric content of food regardless of taste (Burke and Waddell, 2011; Dus et al., 2011; Fujita and Tanimura, 2011; Miyamoto et al., 2012).

Within the "melanogaster group", *Drosophila suzukii* Matsumara (Diptera: Drosophilidae) is a polyphagous insect, with a broad climate range tolerance

and high invasive potential. Unlike *D. melanogaster* that lays eggs and feeds only on decaying and rotten fruits, *D. suzukii* lays eggs and feeds on unripe and undamaged fruits (Dreves, 2011; Rota-Stabelli et al., 2013; Walsh et al. 2011). This difference in ecology is reflected in neurological and physiological adaptations to finding, and feeding on, unripe food sources (Ometto et al., 2013).

On the basis of these considerations, the aim of this study was to evaluate peripheral sensitivity and palatability of different carbohydrates and assess their nutritional value, in adult insects of *D. suzukii* by means of an electrophysiological and behavioural approach. First, the spikes activity was recorded from the labellar l-type sensilla following stimulation with metabolizable mono- and disaccharides (maltose, sucrose, trehalose, glucose, fructose) and non-metabolizable sugars (arabinose, sucralose); second, the palatability of the same sugars was evaluated by recording the proboscis extension reflex (PER) activity; finally, the nutritional value of the carbohydrates was assigned by means of survival trials.

Materials & Methods

Insects

Four to ten-day adults of *Drosophila suzukii* (Diptera: Drosophilidae) were obtained from lab-reared colony at the Dept. of Biomedical Sciences of the University of Cagliari (Italy). In the larval stage, flies were fed on

Drosophila standard diet (Dalton et al., 2011) under controlled conditions (23°C, 70% of relative humidity, 14L/10D photoperiodic regime).

Stimuli

Taste solutions were prepared immediately before testing and were presented at room temperature (23°C). The following sugars were tested: maltose, sucrose, trehalose, glucose, fructose, arabinose, sucralose. They were added with tricholine citrate to provide adequate conductivity to the stimulating/recording solution. All compounds were purchased from Sigma-Aldrich, (Italy).

Electrophysiological experiments

Electrophysiological recordings were performed from the apical pore of the l-type labellar sensilla, by means of the "tip-recording" technique (Fig. 1) (Hodgson et al., 1955). Recording operations were carried out by means of micromanipulators under the field of a stereomicroscope. The reference electrode, a thin Ag/AgCl, was inserted into the base of the isolated head to fix the labellum in prognathous position. The recording electrode, a glass micropipette (tip diameter 20 μm), filled with the stimulating solution, was placed over the sensillum tip. All signals were recorded with a high input impedance (10^{15} Ω) electrometer (WPI, Duo 773), band-pass filtered (0.1 - 3

KHz), digitized by means of an Axon Digidata 1440A A/D acquisition system (sampling rate 10 KHz) and stored on PC for later analysis.

Stimuli were applied in a randomized sequence and a 3-min interval was allowed between consecutive stimulations to minimize adaptation phenomena. All taste stimuli were presented at 100 mM and were dissolved in 30 mM tricholine citrate (TCC), which was also tested alone as control (Charlu et al., 2013; Wieczorek and Wolff, 1989). The 30 mM TCC (control solution) was tested at the beginning and the end of each recording sequence to check for shifts in responsiveness. In order to avoid any drift in solution concentration due to evaporation, a clean, dry piece of filter paper was used to draw fluid from the tip of the recording/stimulating electrode just before each recording. After each test, the labellum was rinsed with distilled water and blotted dry. Finally, we recorded only from one sensillum per fly (N=30-35; 2-3 sensilla/fly) and no preparation was used in more than one experiment.

Data analysis

Recordings typically lasted 2-3 s, but spike analysis was performed in the interval 10-1010 ms after contact with the sensillum, the first 10 ms being skipped as containing the contact artifact. The first second of the discharges was chosen as representative of the phasic/phasic-tonic sections of the response (Dethier and Crnjar, 1982; Inoue et al., 2009). The spike sorting

and counting were performed by means of the Clampfit 10.0 software, based on earlier studies (Dolzer et al., 2003; Dulcis and Levine, 2005; Pézier et al., 2007; Sollai et al., 2014).

Behavioural experiments

Proboscis Extension Reflex (PER)

PER experiments were performed according to Dahanukar et al. (2007) and Burke et al. (2011). Briefly, flies were food deprived for 24 hr inside vials in the presence of water. Flies were trapped into a p200 pipette with the tip cut to expose the head and forelegs to stimuli (Fig. 2). Each tip was held upright on a slide by a piece of clay and was positioned under a stereomicroscope. After 5 min, each fly was observed through the objective of the microscope, and its PER responses were counted. A piece of filter paper was moistened with the sugar solution (100 mM) dissolved in bidistilled water and was brought in contact with the labellar sensilla for 2 s. The sequence of stimulations included a negative control (water), a sugar stimulus and a positive control (2M sucrose). Test stimuli were presented 3 times per fly and each fly was exposed to only one of the test compounds (N=20 flies/sugar). Flies that showed PER to water alone or that failed to extend to 2M sucrose at the end were discarded from the analysis. PER responses were scored as follow: full extension = 100, half or weak extension = 50, no extension = 0.

Survival trials

Immediately after eclosion, flies were separated in 7 groups and each of them was fed on the standard diet that varied only by the sugar type. Each diet contained a sugar amount equivalent to that of the sucrose present in the standard diet (43.82 mM). For each diet, 50 flies per three repeats were allowed to feed ad libitum for 72h e then were divided into 5 vials containing only water (10 flies/vial) (Fig. 3). The number of dead insects was counted every 12h from the beginning of the trial, for a period of 48h.

Statistical analysis

One-way ANOVA was used to analyze: a) the effect of sugar on spike frequency in the first second of discharges; b) the effect of sugar on the activity of the Proboscis Extension Reflex (PER). Two-way ANOVA was used to analyze the interaction of Feeding substrate X Time on flies survival.

Data were checked for the assumptions of homogeneity of variance and normality. Post-hoc comparisons were conducted with the Tukey test, unless the assumption of homogeneity of variance was violated, in which case Duncan's test was used. Statistical analyses were performed using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). P values < 0.05 were considered significant.

Results

Taste sensitivity to sugars

Samples of the GRN spike activity, recorded from the l-type labellar sensilla, in response to different carbohydrates are shown in the figures 4 and 5.

By measuring the peak-antipeak spike amplitude we identified three different spike types that were labelled as small (S), medium (M) and large (L), in response to sugar solutions and two spike types (S, M) in response to TCC alone, added as a conducting agent to all stimuli (Figs. 6, 7).

To test the effect of different stimuli, we analyzed the spike activity evoked in the first second of the discharges to 100 mM of each sugar, by using one-way ANOVA (Fig.8).

One-way ANOVA showed a significant effect of the stimulus on the frequency of the L-type spike ($F[6,304]=95.919$; $p<0.00001$). Post-hoc comparisons showed that the L-type spike frequency in response to disaccharides maltose and sucrose was higher than in response to disaccharide trehalose and to all monosaccharides ($p<0.0001$ for both sugars; Duncan's test), and that spike frequency in response to sucralose was lower than in response to all other sugars tested ($p<0.0001$; Duncan's test). These findings, together with the analysis of spike traces (Figs. 4, 5), indicate that, in the l-type labellar sensilla, the sugars activate only one

GRN and that the effectiveness of the sugars tested is: maltose = sucrose > trehalose = fructose = glucose > arabinose > sucralose.

Effect of sugars on Proboscis Extension Reflex

One-way ANOVA was used to test quantitative differences in the activity of proboscis extension reflex following stimulation of the labellar sensilla with the various sugars tested (Fig. 9): a significant effect of the stimulus on PER activity ($F[6,377]=7.8544$; $p<0.000001$) was found. Post-hoc comparisons revealed flies displayed high levels of PER in response to maltose and sucrose as compared to all other sugars ($p<0.01$), but were not statistically from each other ($p>0.05$; Duncan's test). By contrast, sucralose elicited a significantly lower PER as compared to all other sugars except for fructose ($p<0.05$; Duncan's test), thus eliciting a weak or no response from the labellar sensilla. Finally, trehalose, fructose, glucose and arabinose all elicited PER levels that were statistically indistinguishable from one other. These findings indicate that the palatability to sugars tested is: maltose = sucrose > trehalose = fructose = arabinose = glucose > sucralose.

Effect of sugars on fly survival

Mean values ± s.e.m. of the number of survived flies on each feeding substrate (standard diet with 43.82 mM of one of the test sugars) are shown in figure 10. Two-way ANOVA revealed a significant interaction of

Feeding substrate x Time on fly survival ($F[24,70]=17.552$; $p<0.00001$). In the case of disaccharides, post-hoc comparisons showed that the number of flies fed and survived on standard diet containing maltose and trehalose was significantly lower compared to the control (end of feeding and start of starvation) after 48h and 36h fasting, respectively ($p<0.01$; Duncan's test), and already after 24h for the flies fed and survived on standard diet containing sucrose ($p<0.05$; Duncan's test). Moreover, we found that the number of flies survived after fasting for 24, 36, 48h was significantly lower if they were fed on sucrose than those fed on maltose and lower than those fed trehalose after 24, 36h ($p<0.005$; Duncan's test) (see Tables 1-4).

These findings indicate that flies fed on a diet containing maltose or trehalose have a significantly longer lifespan compared to flies on sucrose diet, thus suggesting a higher nutritional value for the first two disaccharides.

For the monosaccharides, post-hoc comparisons showed that the number of flies fed and survived on standard diet containing fructose, glucose, arabinose or sucralose was significantly lower compared to the control already after 24h fasting ($p<0.0001$; Duncan's test). Besides, the number of flies fed and survived on sucralose and arabinose was significantly lower compared to all other monosaccharides and survival success on arabinose was lower than on fructose or glucose, respectively, already after fasting for 24h ($p<0.05$; Duncan's test); instead, no difference was found between the

number of flies fed and survived on fructose and glucose, at all time checks (24, 36, 48h; $p>0.05$; Duncan's test) (see Tables 1-4).

These findings indicate that flies fed on a diet containing sucralose or arabinose have a significantly shorter lifespan compared to fructose and glucose and this suggests a lower nutritional value for the first two monosaccharides.

Discussion

The main role of the taste system is to provide information about food, palatability, by allowing insects to recognize and evaluate both the presence of an energy source such as carbohydrates and/or potentially harmful bitter compounds, that respectively produce an appetitive or aversive feeding behaviour (Fujita et al., 2011; Miyamoto et al., 2012; Sollai et al., 2014; Sollai et al., 2015). It is known that feeding behaviour is mainly affected by two factors: food palatability and the nutritional needs (Dus et al., 2011).

In this study we have first investigated in *Drosophila suzukii* the presence of differences in the taste sensitivity of labellar sensilla both as spike frequencies and levels of PER, that represents an effective measure of palatability (Stafford et al., 2012). We found that the disaccharides maltose and sucrose evoked higher spike frequencies and induced higher PER responses, suggesting that they strongly activate the sweet-sensing GRN and are the more palatable sugars. On the contrary, sucralose elicited very low

spike frequencies and reflex responses, proving to be a weaker stimulus for the sugar-sensitive GRN and a less palatable sugar. Finally, trehalose, fructose, glucose and arabinose all elicited intermediate spike frequencies and levels of PER. These results are consistent with previous studies on other insects. In *Phormia regina* the ranking of stimulating effectiveness of sugars is maltose = sucrose > trehalose for the disaccharides and fructose > glucose = arabinose for the monosaccharides (Hassett et al., 1950). Data from several laboratories about *D. melanogaster* show that: glucose and arabinose evoke both spike frequencies and levels of PER of similar strength (Fujita et al., 2011); the flies respond most robustly to disaccharides such as sucrose and maltose, while fructose and arabinose elicit intermediate levels of PER (Dahanukar et al., 2007; Lee et al., 2014; Liman et al., 2014; Stafford et al., 2012).

Taken together, our results on spike frequencies evoked and on the levels of PER activity, show that taste sensitivity and palatability for the sugars are in close agreement with each other. In fact, we found that those sugars that evoke a higher number of spikes are also those that promote a higher activity of PER, thus suggesting that the more stimulant is a sugar the more appetitive it is for flies. A positive relationship between electrophysiological recordings and PER activity has already been described in *D. melanogaster* (Fujita et al., 2011).

Still a matter of debate is whether taste sensitivity and palatability of sugars are an indicator of their nutritional value (Burke et al., 2011; Dus et al., 2011; Fujita et al., 2011; Linford et al, 2015; Miyamoto et al., 2012; Stafford et al., 2012). Previous studies about *P. regina* suggest that there is no direct relationship between the taste of a compound and its nutritional value: sugars exist that are very stimulating, but have no nutritional value, and vice versa (Hassett et al., 1950). Most studies on *D. melanogaster* show that initially flies choose a sugar in strict accordance with taste or palatability; however, following deprivation of food, their preference shifts toward sugars with a higher nutritional content, sensed through postingestive mechanisms (Burke et al, 2011; Dus et al., 2011; Fujita et al., 2011; Stafford et al., 2012). Instead, other studies suggest that taste sensitivity plays an important role in determining appropriate physiological and behavioural responses to the availability of nutrients, particularly when flies live in environments where they encounter food sources with low nutritional contents (Linford et al., 2015). In agreement with other reports, we found that flies fed on diets containing metabolizable sugars (maltose, sucrose, trehalose, glucose and fructose) live longer than those fed on diets with non-metabolizable sugars (arabinose and sucralose) (Lebreton et al., 2014; Lee et al., 2014; Stafford et al., 2012). Moreover, the survival capability is longer for flies fed with disaccharides, in particular maltose, than for those fed with monosaccharides (Hassett et al., 1950). In general,

we found a partial discrepancy between the nutritional value of sugars and the responsiveness of sweet-sensitive GRN. In fact, on the one hand flies fed with maltose (the more stimulating and palatable sugar) are those the live longer; while, flies fed with sucralose (the less stimulating and palatable sugar) have a shorter life. On the other hand flies fed with trehalose survive longer than those reared on sucrose, that elicits a higher responsiveness; besides, flies fed with arabinose survive for a shorter time than those reared on glucose and fructose, even though the GRN stimulating effectiveness and levels of PER were statistically indistinguishable.

At the moment we cannot fully explain these results: in fact, do flies fed for 72h on a diet containing disaccharides (such as maltose) survive longer than those reared on a diet with a monosaccharides because they ingest more sugar, along with its higher palatability, and/or because the sugar has a higher nutritional value, according to its caloric content? In *D. melanogaster* it is suggested that flies possess an internal nutrient sensor that regulates food consumption based on the nutritional value of carbohydrates; this sensor evaluates hemolymphatic fructose levels, which can be derived either directly from fructose in the diet or from other nutritious sugars broken down or, again, metabolized from glucose, modifying the feeding behaviour in a satiation-dependent manner (Miyamoto et al., 2012). If this holds true also for *D. suzukii*, we may suppose that flies ingest less sucrose because its digestion readily yields fructose, while in the case of maltose and trehalose

(both formed by two glucose molecules) the hemolymphatic levels of fructose rise slowly as glucose monomers must be metabolized. By contrast, given the fact that arabinose and sucralose are non-metabolizable sugars, they sustain less efficiently the survival capability of flies, thus suggesting that they possess little or no nutritional value (Lebreton et al., 2014; Stafford et al., 2012).

Further investigations are underway to determine whether the differences in the number of surviving insects for each control are due to the amount of food eaten or to the ability to metabolize the different carbohydrates, for example by turning them into fat that can provide energy for longer times, or both possibilities.

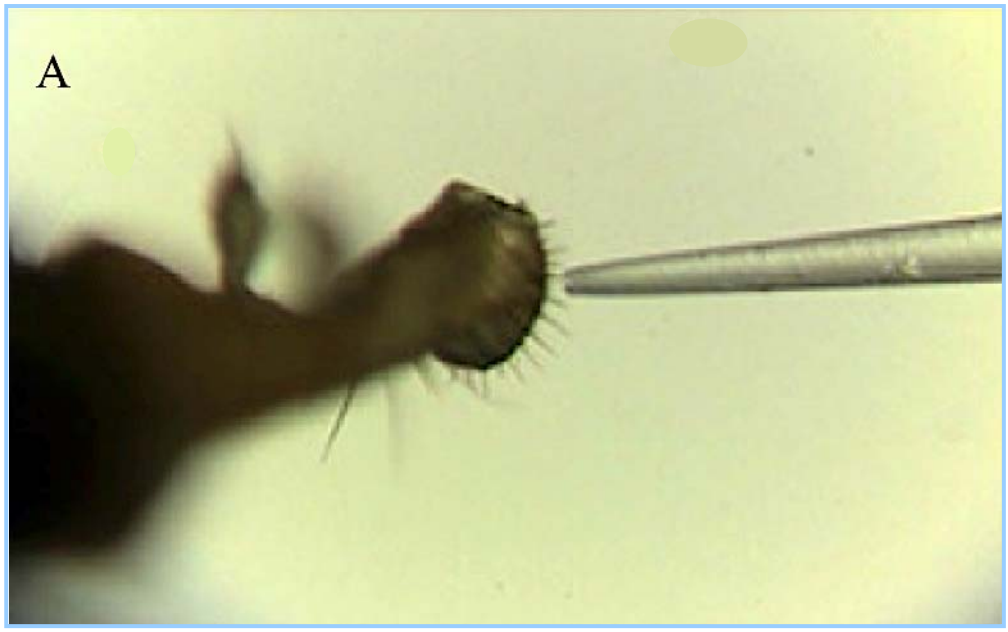
Figures

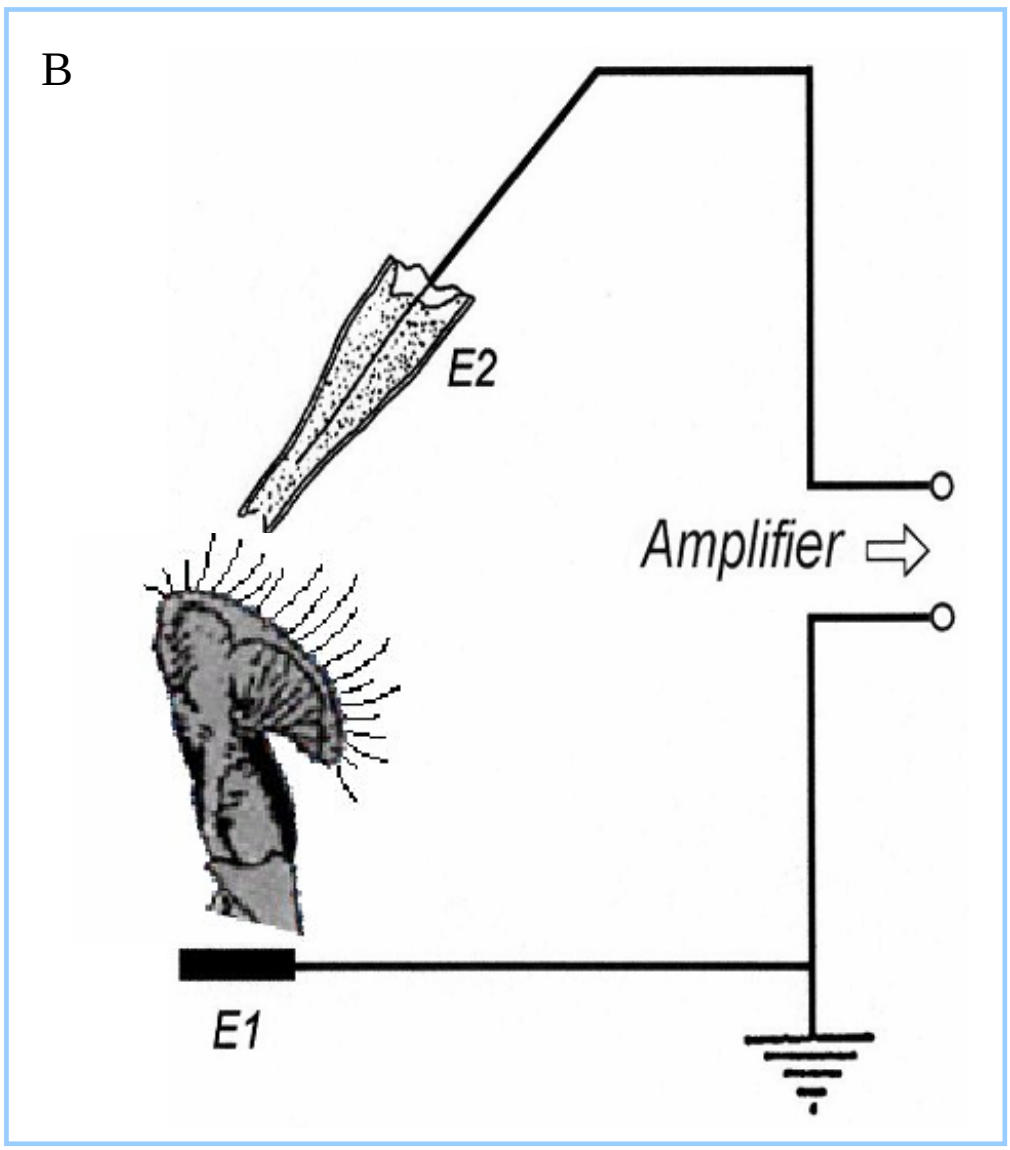
Figure 1 – Recording technique

Picture (A) and lay-out (B) showing the “tip-recording” technique:

E1= reference electrode;

E2= recording/stimulating electrode.


A


B
E2
Amplifier
E1

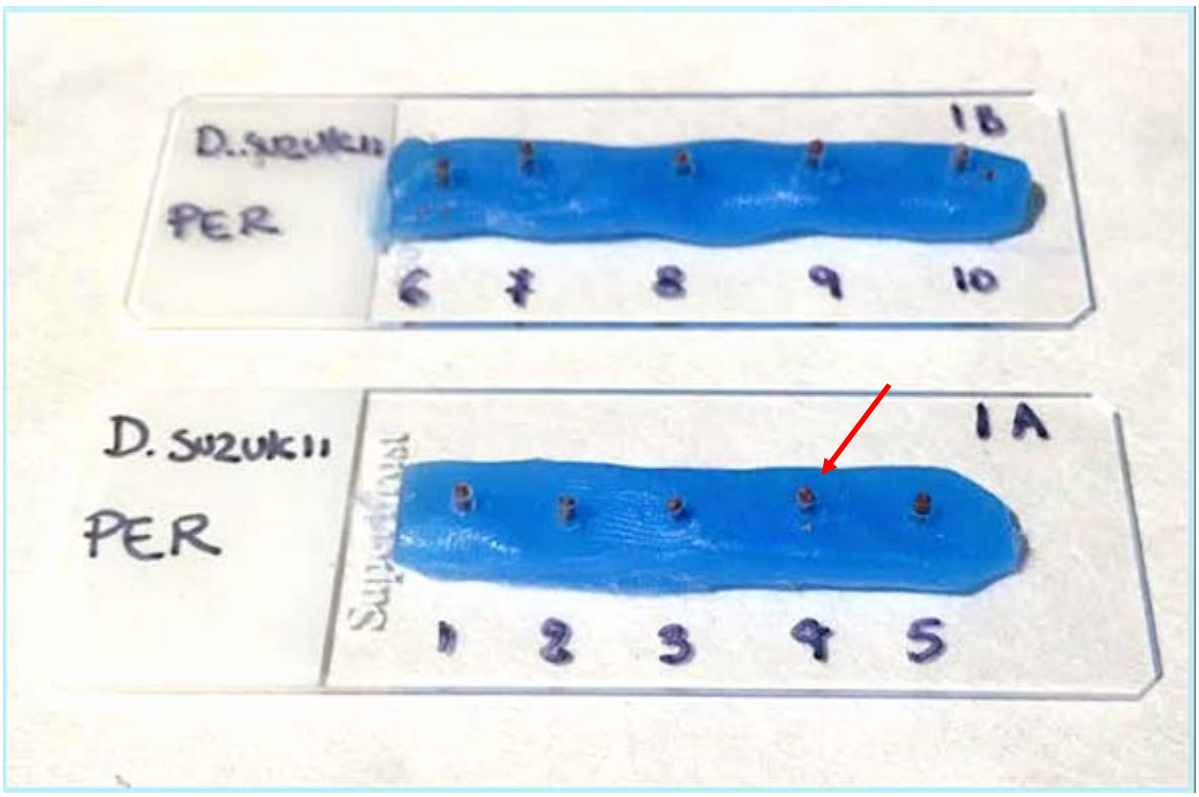


Figure 2 - Behaviour

Experimental arena for the PER trials.

Red arrow indicates one of the p200 pipettes from which the head of the flies protrude.

Figure 3 – Behaviour

Experimental arena for the survival trials showing 5 vials, each containing 10 flies at the start of the starvation test.

Figure 4 – Sample of spike discharges

Sample traces showing spike activity of a labellar l-type sensillum following stimulation with TCC (control), maltose, sucrose and trehalose.

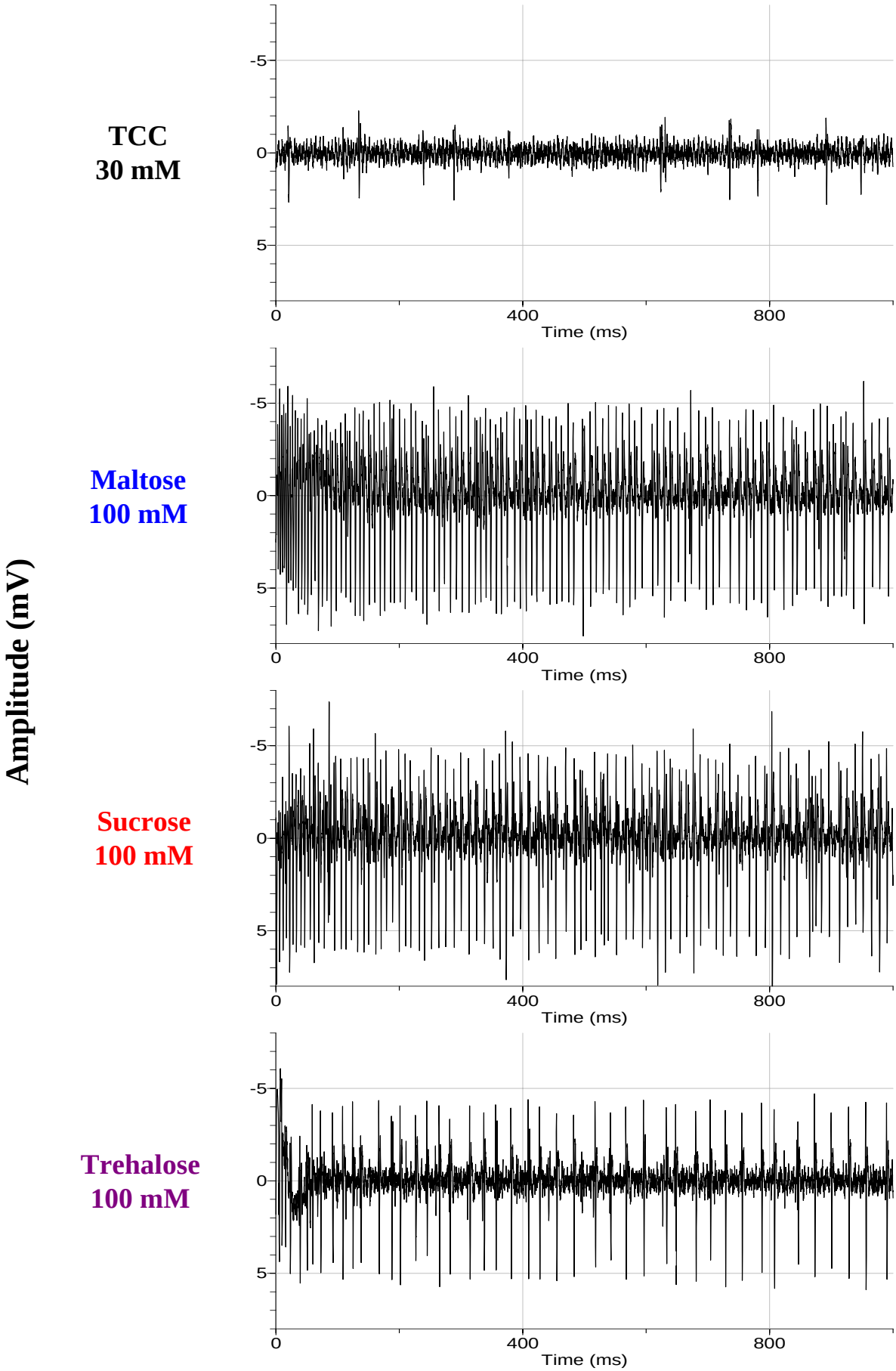

TCC
30 mM
Maltose
100 mM
Sucrose
100 mM
Trehalose
100 mM
Amplitude (mV)
-5
0
5
0
400
800
Time (ms)

Figure 5 – Sample of spike discharges

Sample traces showing spike activity of a labellar l-type sensillum following stimulation with glucose, fructose, arabinose and sucralose.

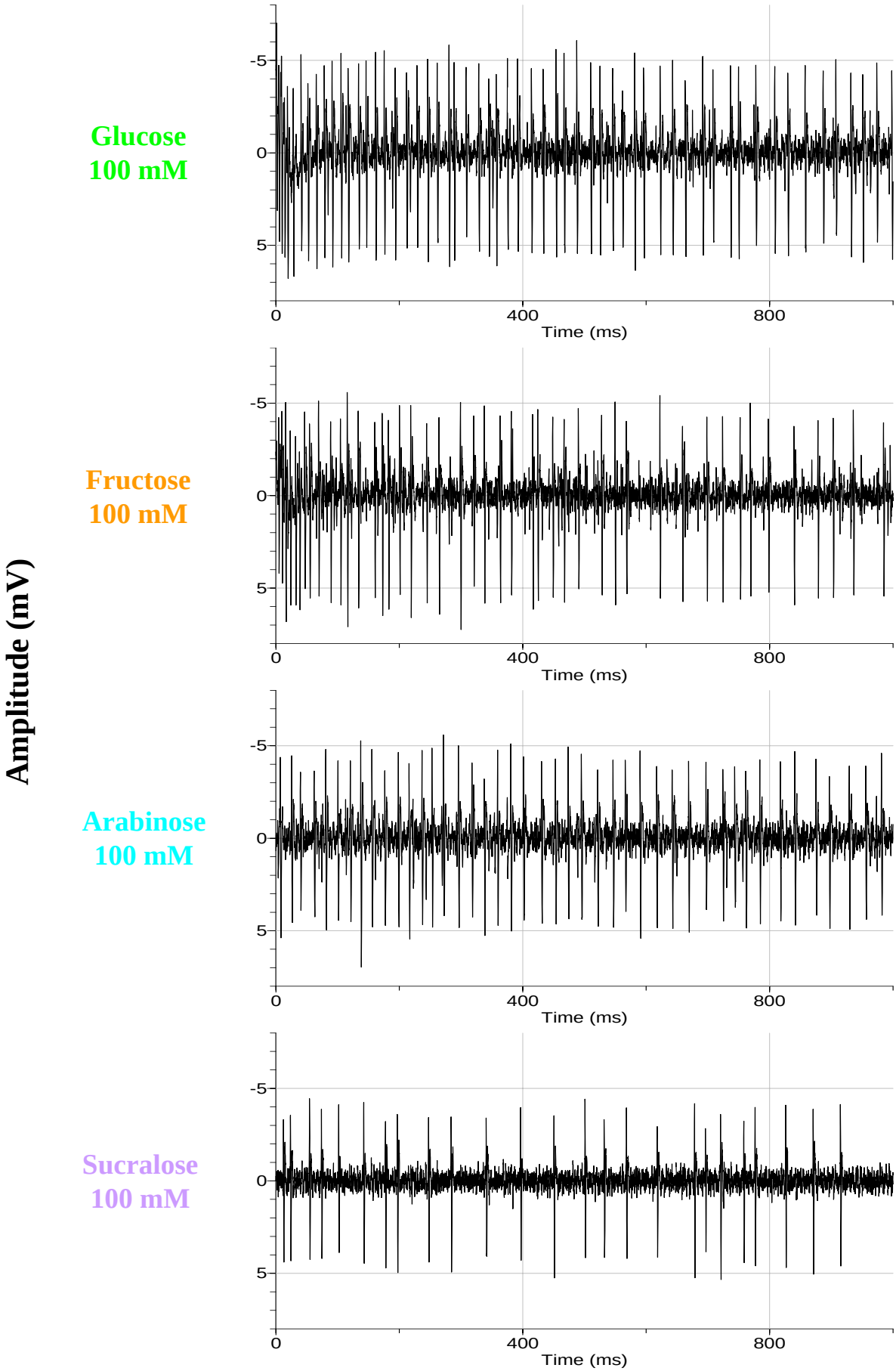
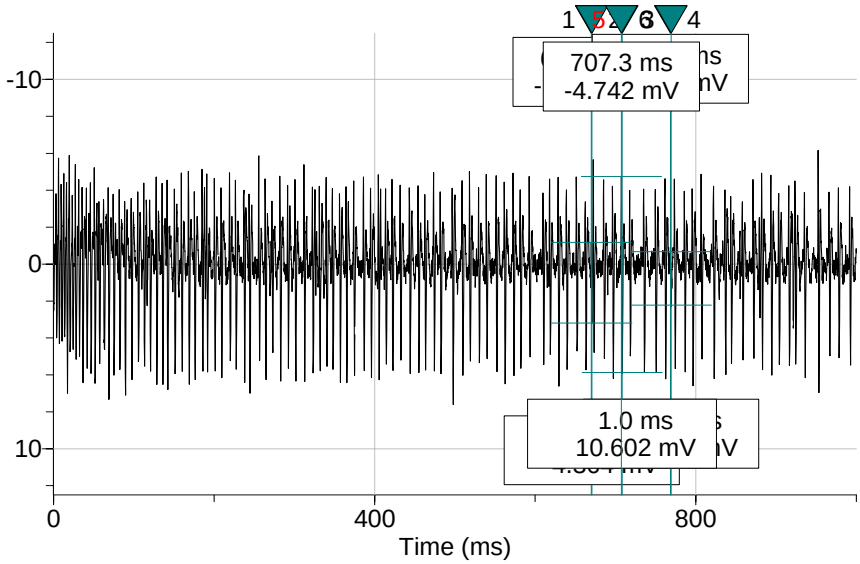
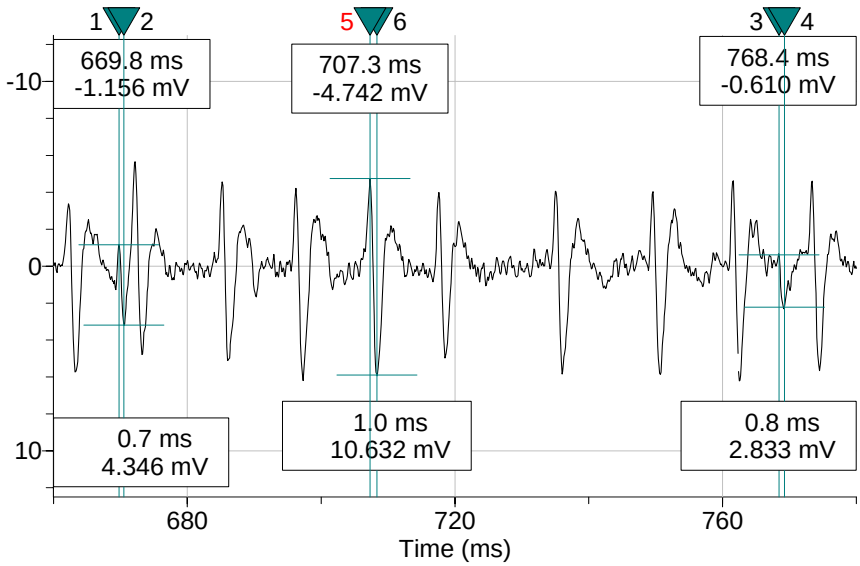

Glucose
100 mM
Fructose
100 mM
Arabinose
100 mM
Sucralose
100 mM
Amplitude (mV)
-5
0
5
0
400
800
Time (ms)

Figure 6 - Spike identification by amplitude

Spike discharge (A) and a portion thereof expanded (B) showing three different spike types, S, M and L, in response to 100 mM maltose. Spike amplitude classes are given in the histogram (C). Vertical red dashed lines are the ideal boundaries of the spike types.


707.3 ms
-4.742 mV
1.0 ms
10.602 mV
-10
0
10
0
400
800
Time (ms)


669.8 ms
-1.156 mV
707.3 ms
-4.742 mV
768.4 ms
-0.610 mV
0.7 ms
4.346 mV
1.0 ms
10.632 mV
0.8 ms
2.833 mV
-10
0
10
680
720
760
Time (ms)

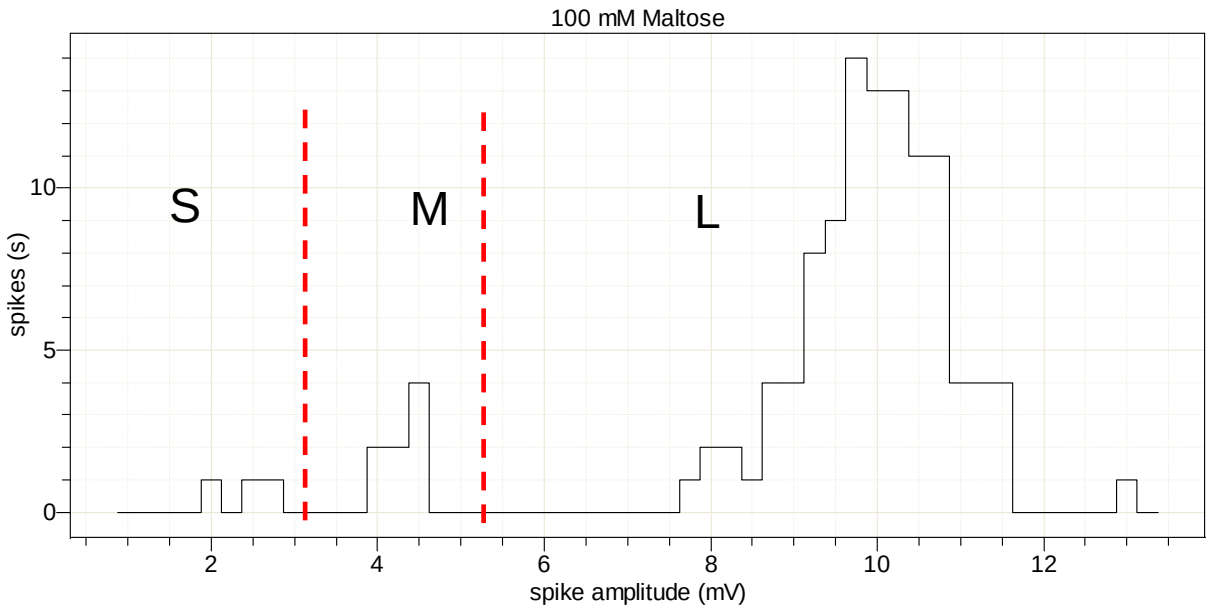
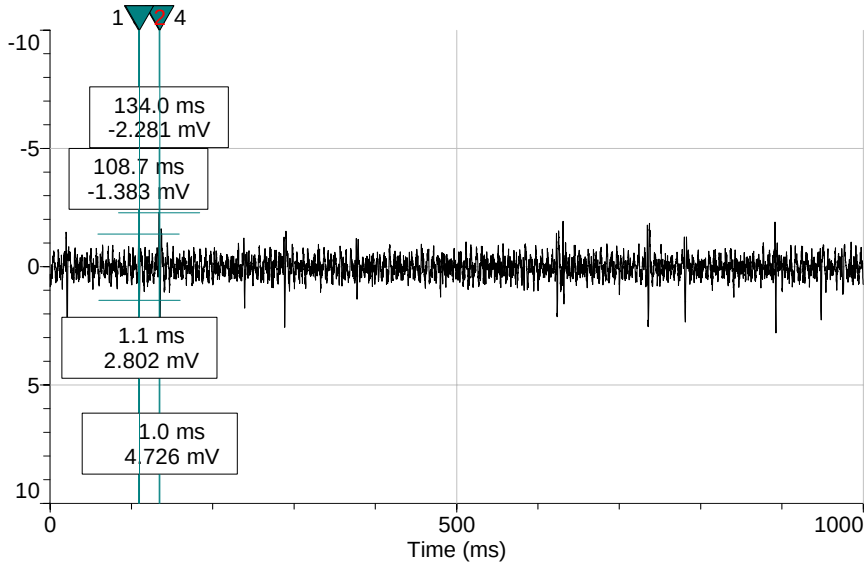
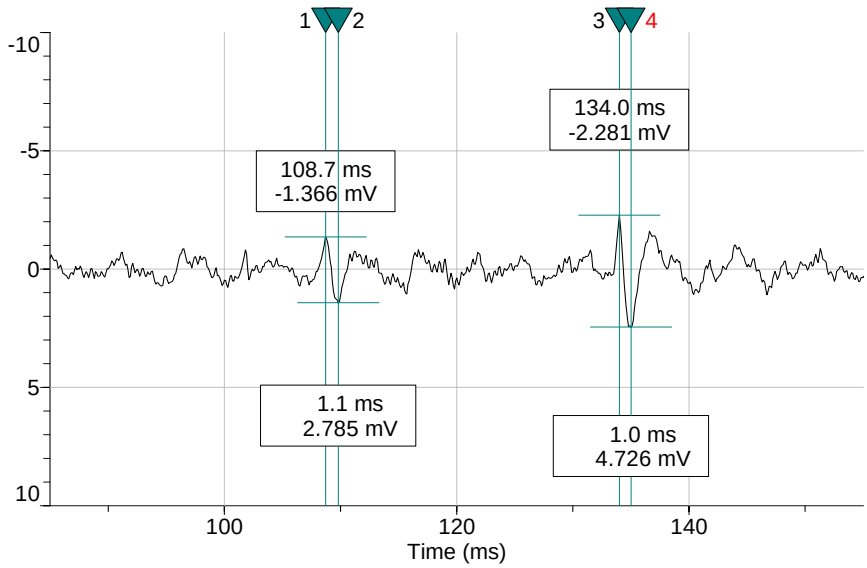
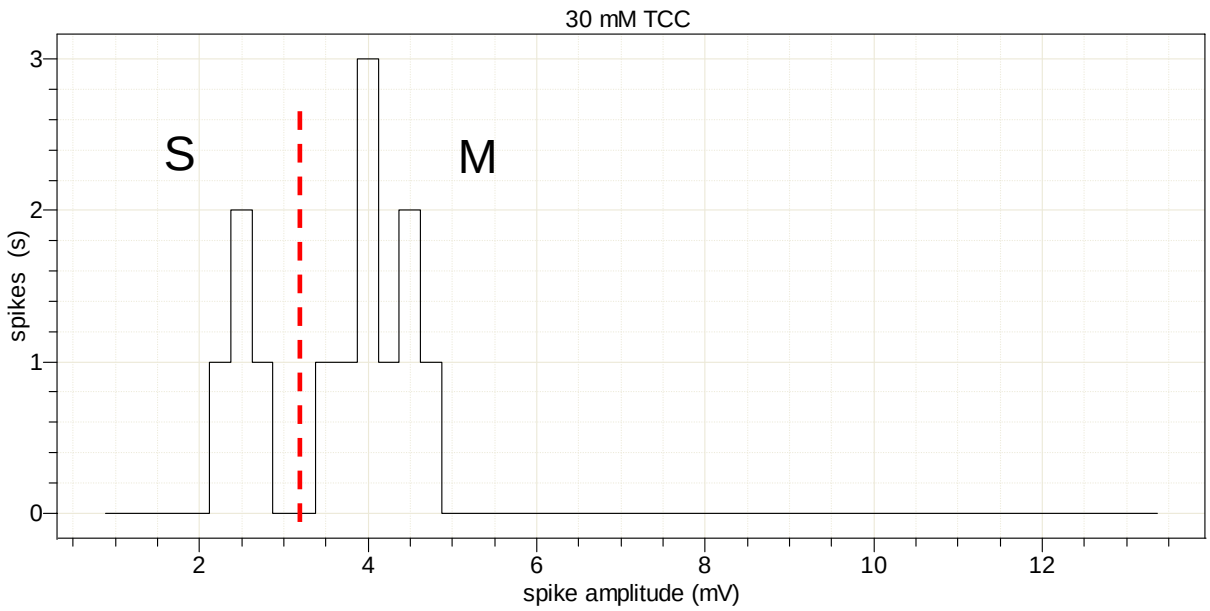

100 mM Maltose
S
M
L
spikes (s)
10
5
0
2
4
6
8
10
12
spike amplitude (mV)

Figure 7 - Spike identification by amplitude

Spike discharge (A) and a portion thereof expanded (B) showing two different spike types, S and M, in response to 30 mM TCC. Spike amplitude classes are given in the histogram (C). Vertical red dashed line is the ideal boundary of the spike types.


1
2
4
-10
-5
0
5
10
134.0 ms
-2.281 mV
108.7 ms
-1.383 mV
1.1 ms
2.802 mV
1.0 ms
4.726 mV
0
500
1000
Time (ms)


1
2
3
4
-10
-5
0
5
10
108.7 ms
-1.366 mV
134.0 ms
-2.281 mV
1.1 ms
2.785 mV
1.0 ms
4.726 mV
100
120
140
Time (ms)


30 mM TCC
S
M
spikes (s)
0
1
2
3
2
4
6
8
10
12
spike amplitude (mV)

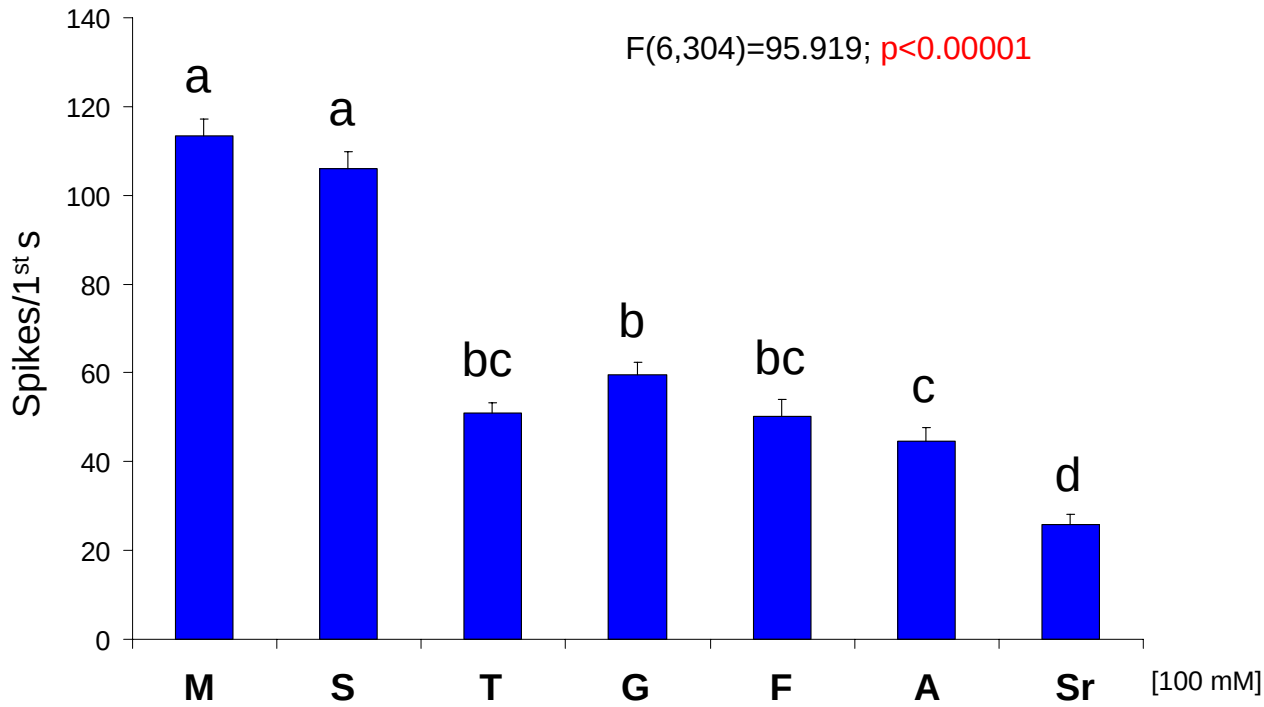


Figure 8 – Electrophysiological results

Mean values ± s.e.m. of spike frequency of "sugar" GRN following stimulation with 100 mM maltose (M), sucrose (S), trehalose (T), glucose (G), fructose (F), arabinose (A) and sucralose (Sr). N= 30-35 (2-3 sensilla/fly). Different letters indicate significant differences ($p<0.005$; Duncan's subsequent to one-way ANOVA).

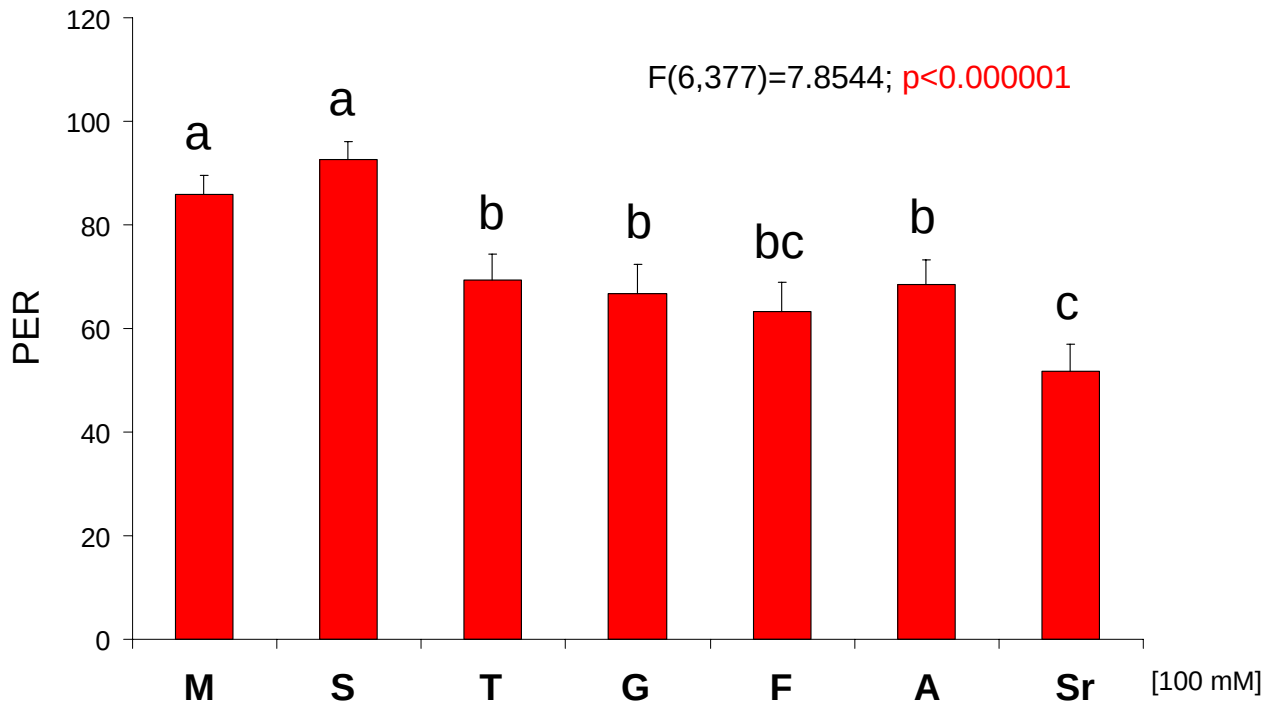


Figure 9 – PER results

Mean values ± s.e.m. of PER activity following stimulation of labellar sensilla with 100 mM maltose (M), sucrose (S), trehalose (T), glucose (G), fructose (F), arabinose (A) and sucralose (Sr). N=20 flies/stimulus. Different letters indicate significant differences. ($p<0.05$; Duncan's test subsequent one-way ANOVA).

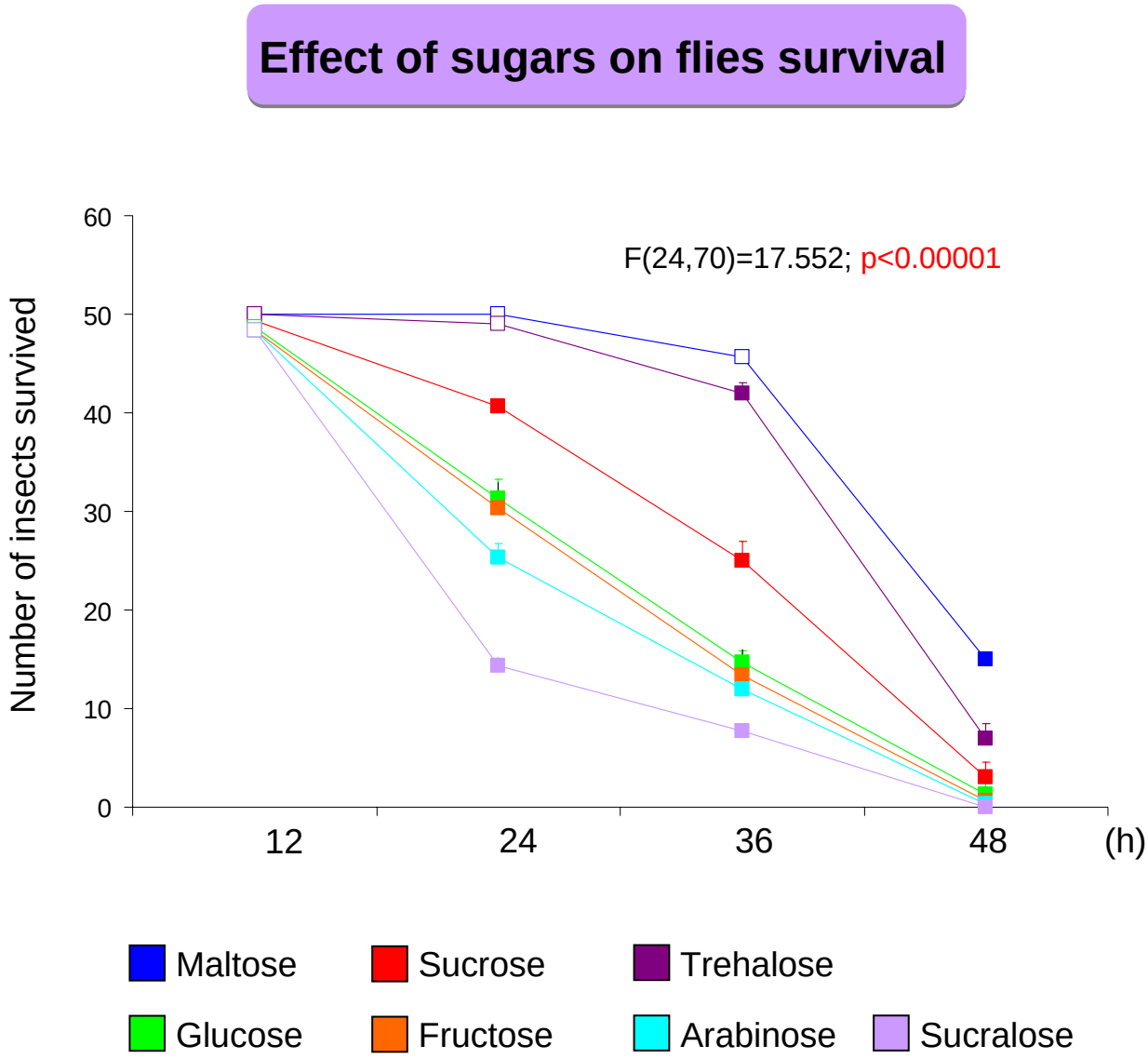
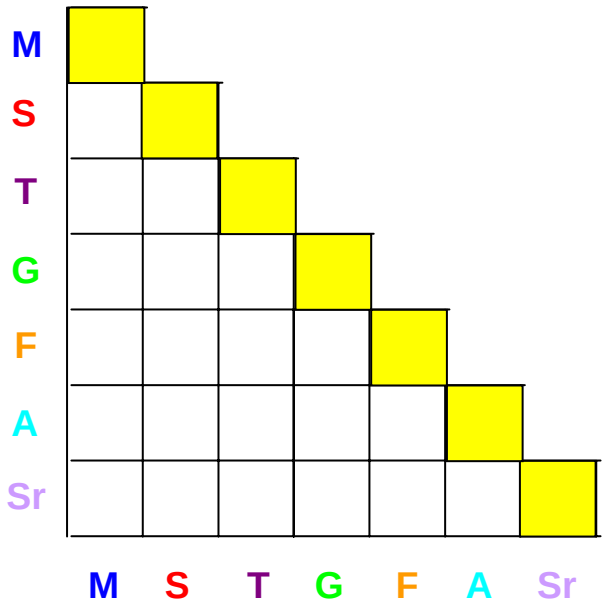


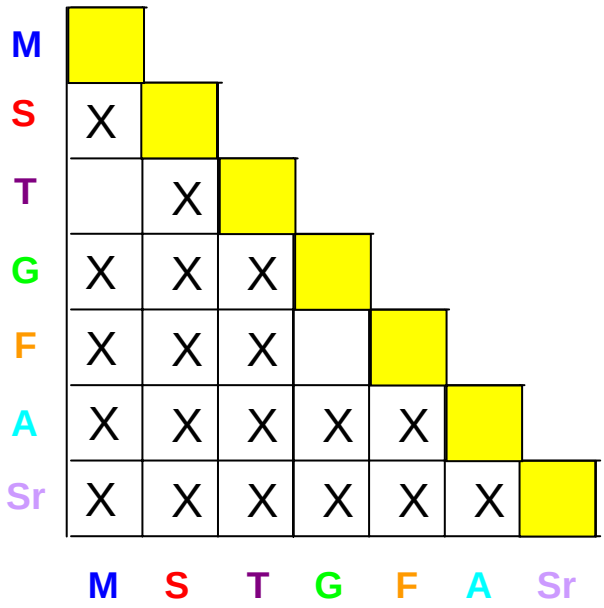
Figure 10 – Survival results

Mean values ± s.e.m. of the number of flies survived on each feeding substrate after 12, 24, 36 and 48 h fasting. Filled symbols indicate significantly different from control (number of insects at the end of feeding and start of starvation) ($p<0.05$; Duncan's test subsequent to two-way ANOVA).

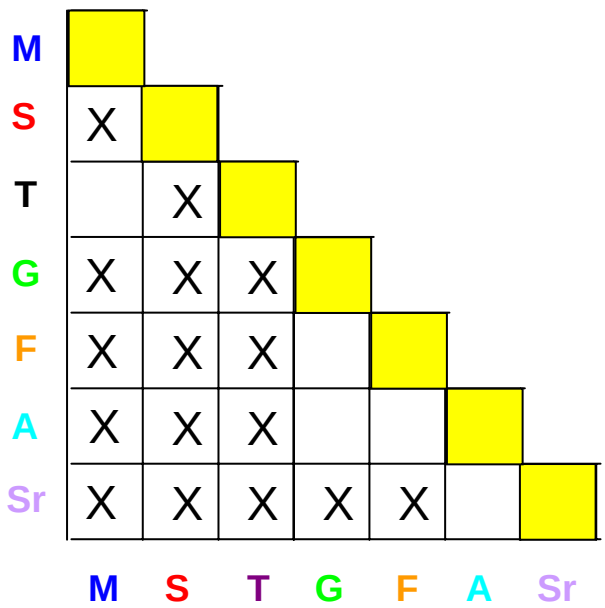
Tab. 1 –12h



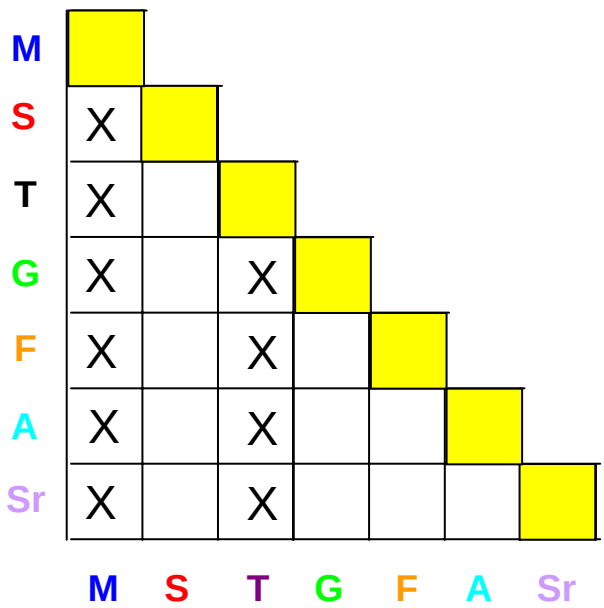
Tab. 2 –24h



Tab. 3 –36h



Tab. 4 –48h



Tables 1-4 - Survival results

X = significant differences of the number of flies survived between pairs of substrates (e.g., between maltose and sucrose) at each time check (12, 24, 36 and 48 h starvation) ($p<0.05$; Duncan's test subsequent to two-way ANOVA).

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