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Marie-Jeanne Sellier

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Modulation of feeding behavior and peripheral taste response by aversive molecules in *Drosophila melanogaster*

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I. General introduction

1. Mechanisms evolved by the insects to cope with the secondary plant compounds

Insects' co-evolution with the vegetal kingdom has led to the development of costly defense mechanisms by plants, as a protection against herbivores. Among those mechanisms, we can find the induction of defensive proteins (Haruta *et al.*, 2001), the release of volatiles to attract predators or parasitoids of the herbivorous insects (Birkett *et al.*, 2000; Birkett *et al.*, 2003) or the production of secondary compounds (Baldwin, 2001; Kliebenstein *et al.*, 2001). Terrestrial plants produce a diverse array of secondary metabolites, likely more than 100,000 molecules and at least some of them have a role in the defense of the plant against herbivores (Schoonhoven, 1982). Plant chemical composition is variable and represents a challenge for insect feeding. Most plant defensive chemicals discourage insects, either by deterring feeding and oviposition or by impairing larval growth, rather than by killing insects outright. Indeed, deterrence and toxicity are not necessarily closely coupled (Bernays, 1991; Glendinning, 1996) and there are high interspecific differences in bioactivity of the antifeedants (González-Coloma *et al.*, 2002).

Insects have developed strategies to cope with those secondary compounds. Preingestive mechanisms prevent them from consuming high quantities of potentially toxic food. First, herbivorous insects have taste cells responding to unpalatable and/or toxic plant compounds and each species has a unique sensory window, which can discriminate between host and non-host plants (Schoonhoven, 1982; Glendinning, 2002). Insects may not be able to discriminate among different bitter compounds based on chemical identity, but might distinguish compounds within a modality based on intensity or palatability (Masek and Scott, 2010). On the other hand, different compounds may be processed by different signaling pathways, which could lead to discrimination among molecules (Glendinning *et al.*, 2002). Secondly, the presence of carbohydrates can increase the palatability of some deterrent compounds by masking their unpleasant taste, which allows the insect to override its aversive response in some cases (Shields and Mitchell, 1995b; Glendinning *et al.*, 2000). Finally, insects can learn to associate the lipid composition of the surface of the leaves with deterrent compounds present inside the plant (Blaney and Simmonds, 1985). In addition to the sensory detection of toxic molecules, insects

have developed postingestive processes to reduce or abolish the effects of the ingested secondary compounds. Efficient detoxification systems, using mechanisms such as oxidation, reduction, hydrolysis and then conjugation of molecules, allow them to get rid of most of the toxic compounds present in the plants they feed on (Scott and Zhimou, 2001; Després *et al.*, 2007). Some insects can sequester poisons (Nishida, 2002), which reduces the need of detoxifying them and can be used in turn to deter potential predators. Lastly, insects may learn to associate some plants with the adverse postingestive effect they cause, in order to avoid them (Lee and Bernays, 1990).

The food aversion caused by a deterrent compound can decrease following a long or repeated exposure to this molecule. This phenomenon, found in mammals as well as in insects, is called habituation. Habituation is defined as the waning of a response as a result of repeated or prolonged presentation of a stimulus, which is not due to sensory adaptation or motor fatigue (Carew and Sahley, 1986). Habituation differs from sensory adaptation in its ability to be terminated or reversed immediately by a novel or noxious stimulus (Thompson and Spencer, 1966). This phenomenon has been shown in different lepidoptera species such as *Spodoptera litura* (Bomford and Isman, 1996), *Pseudaletia unipuncta* (Usher *et al.*, 1988), *Manduca sexta* (Glendinning *et al.*, 1999; Glendinning *et al.*, 2001b; Glendinning *et al.*, 2001a) or *Trichoplusia ni* (Akhtar *et al.*, 2003) or in the grasshopper *Schistocerca americana* (Glendinning and Gonzalez, 1995). Decreased response to antifeedants following prolonged exposure occurs most readily when a single antifeedant provides a weak inhibitory stimulus (Szentesi and Bernays, 1984). On the other hand, habituation is more difficult when insects are exposed to a mixture of compounds (Bomford and Isman, 1996; Akhtar and Isman, 2003). A decrease in response to feeding deterrents might enable the insect to feed normally on plant species that belong to the potential host-plant spectrum and would permit broadening of diet if the need arises.

Previous studies have shown that prolonged exposure to a deterrent molecule could be associated with a decrease in peripheral taste sensitivity (Glendinning *et al.*, 1999; Bernays *et al.*, 2003) and an increase in the activity of the P450 detoxification enzymes (Bhaskara *et al.*, 2006). Moreover, the induction of P450 enzymes consecutive to the exposure to an antifeedant can cause an increase in the consumption of the usually avoided compound (Glendinning and Slansky, 1995). However, those different observations have never been directly correlated. P450 enzymes are active in different organs of the insects, in the gut in particular. Nevertheless, gustatory neurons are in

regular contact with toxic molecules, as well as the accessory cells they are associated with. Thus, there should be a protection system for these cells. In olfactory sensilla, different proteins bind to the odorants, such as OBP (odorant binding molecules) or degradation enzymes (Vogt *et al.*, 2002). Similar proteins may exist in taste sensilla and P450 enzymes could potentially contribute to the protection of the sensory and accessory cells.

The initial hypothesis we wanted to test in this PhD thesis was a negative correlation between the sensitivity to deterrent molecules and the efficiency of the detoxification systems, with a direct influence on the taste organs' sensitivity. To this end we had planned to use electrophysiological, behavioral, pharmacological and genetical approaches.

In this work, we describe a quantitative multiple-choice feeding test to build dose-response profiles and we use this assay to test 8 alkaloids. We show that high concentrations of these alkaloids reduce the appetitive effect of sugars. Secondly, we find a correlation between our behavioral results and the electrophysiological inhibition of sugar detection by antifeedants in sensilla of the proboscis. We study the hypothesis of a lateral interaction between the bitter- and the sugar-sensing cells to explain this inhibition and we discard this hypothesis. Finally, we describe our attempts to elicit habituation to caffeine in the fruit fly.

2. Morphology and physiology of taste in *Drosophila melanogaster*

Fruit flies react to taste molecules in a way which is quite similar to humans (sometimes more than rodents, see: Gordesky-Gold *et al.*, 2008) and within the detection range of mammals. They are attracted to sugars, avoid bitter and toxic molecules and adapt their consumption of acids and salts to their internal needs (Amrein and Thorne, 2005; Gerber and Stocker, 2007). Although the taste system of mammals and fruit flies is anatomically different, the numerous functional similarities between them, the relative simplicity of the insects' gustatory system and the molecular tools available make *Drosophila melanogaster* a good model to study taste perception (Scott, 2005).

In *Drosophila*, taste is mediated through taste hairs, called sensilla, located on mouthparts, the legs, the wings margin and the ovipositor (Figure 1, Left). Taste sensilla directly influence feeding activities, especially those located on the mouthparts, *i.e.* on the labellum or proboscis.

All taste sensilla have a pore at their tip that let chemicals penetrate the hair shaft and contact the dendrites of four gustatory receptor neurons (GRNs). The sensilla on the labellum are classified into three types according to their length (L: long, S: small and I: intermediate) (Shanbhag *et al.*, 2001) (Figure 1, Right). L- and S-type sensilla house four GRNs responding mainly to water (W-cell), sugars (S-cell), low (L1-cell) and high (L2-cell) concentrations of salts respectively (Rodrigues and Siddiqi, 1981; Fujishiro *et al.*, 1984; Hiroi *et al.*, 2002). In S-type sensilla, the L2 cell also responds to bitter compounds. I-type sensilla only contain two GRNs (Shanbhag *et al.*, 2001), one combining the functions of the S and L1 cells and the other being the L2 cell and responding to aversive molecules (Hiroi *et al.*, 2004). The axons of the labellar GRNs directly project to interneurons in the subesophageal ganglion (Ishimoto and Tanimura, 2004; Wang *et al.*, 2004; Amrein and Thorne, 2005; Miyazaki and Ito, 2010).

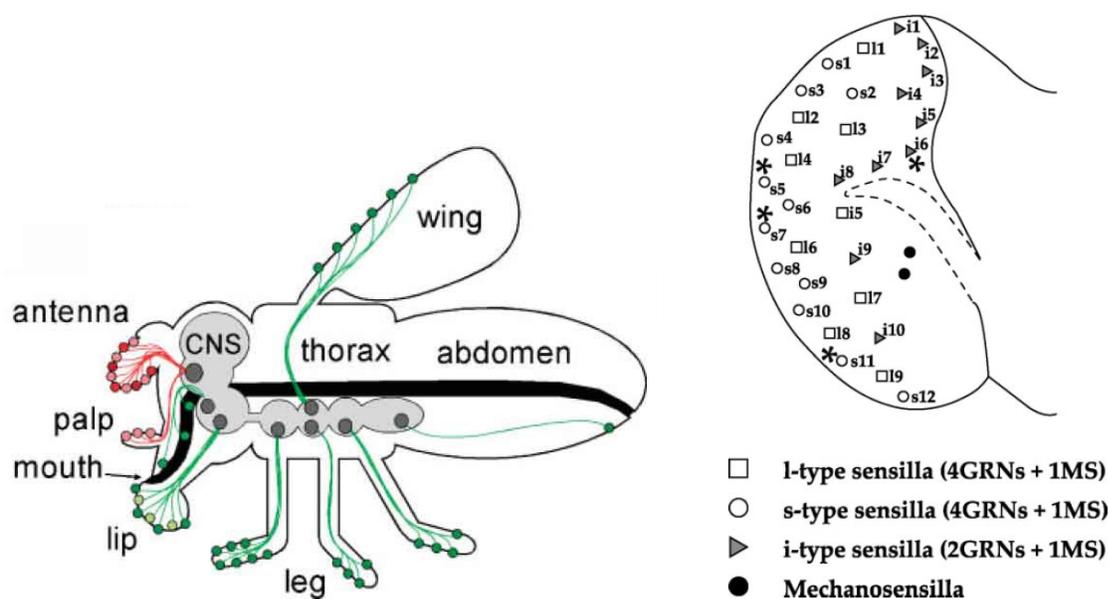


Figure 1. Organization of the taste system in *D. melanogaster*.

Left: Location of the olfactory (red) and gustatory (green) sensilla in *D. melanogaster* (de Bruyne and Warr, 2006). The CNS is in grey and the digestive tract in black. **Right:** Map of the taste sensilla on the labellum of *D. melanogaster*. GRN: Gustatory receptor neuron, MS: Mechanosensory neuron (Hiroi *et al.*, 2004).

A family of 68 candidate gustatory receptors (GRs) has been identified (Clyne *et al.*, 2000; Dunipace *et al.*, 2001; Scott *et al.*, 2001) (Figure 2). While a few of them are known to be involved in sugar or pheromone perception, many others could be involved in the detection of aversive molecules (Amrein and Thorne, 2005). A family of structurally closely related receptor genes is expressed in the sugar-sensing cells: *Gr5a*, *Gr64a-f* and *Gr61a* (Jiao *et al.*, 2007). GR5a and GR64a appear to be the main sugar receptors (Dahanukar *et al.*, 2007) and GR64f could be a required co-receptor (Jiao *et al.*, 2008). The deletion of *Gr61a* does not seem to affect the electrophysiological response to sugars and its function remains unknown (Dahanukar *et al.*, 2007).

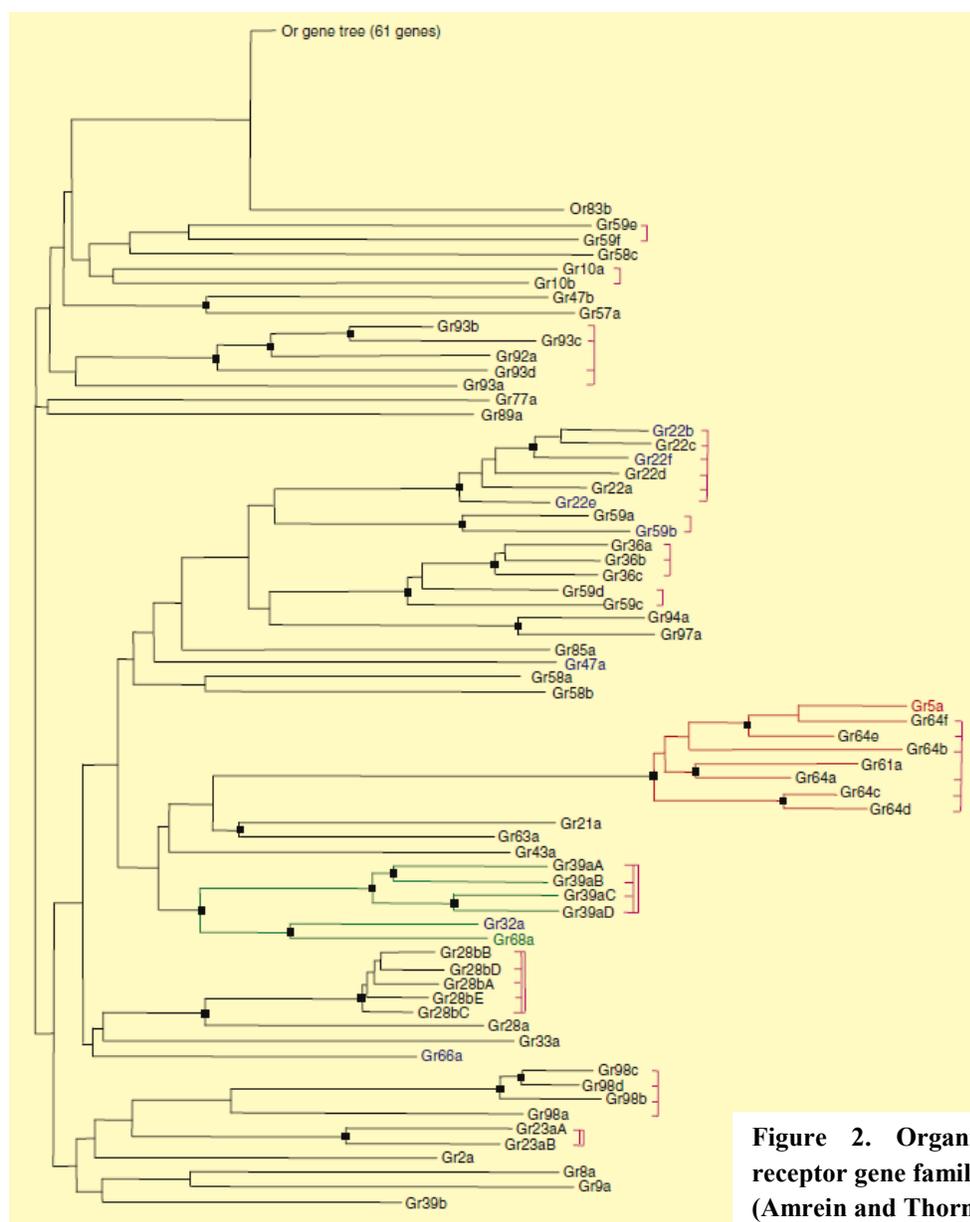


Figure 2. Organization of the gustatory receptor gene family.
(Amrein and Thorne, 2005)

Several studies have shown that *D. melanogaster* was sensitive to bitter substances, especially alkaloids such as quinine, strychnine or caffeine (Meunier *et al.*, 2003; Marella *et al.*, 2006; Moon *et al.*, 2006). These compounds activate GRNs dedicated to aversive taste stimuli, which triggers avoidance behaviors (Meunier *et al.*, 2003; Hiroi *et al.*, 2004; Lacaille *et al.*, 2007). How these cells respond to bitter chemicals and which receptors are involved is still under debate. The most extensively studied case is that of the perception of caffeine. GR66a and GR93a seem to work as co-receptors to detect caffeine and theophylline, but not theobromine, another methylxanthine (Lee *et al.*, 2009). Nevertheless, the misexpression of these two receptors into sugar-sensing cells is not enough to give them the capability to detect caffeine (Moon *et al.*, 2006; Lee *et al.*, 2009). Recently, an additional *Gr* has been shown to be involved in bitter perception: *Gr33a*, which is phylogenetically the closest *Gr* gene from *Gr66a* and which is also expressed in bitter-sensitive cells (Moon *et al.*, 2009). Mutants for *Gr33a* display a reduction of the response to all the tested antifeedants but the other receptors are still expressed in the GRNs' dendrites, which means that *Gr33a* is not involved in receptor trafficking. These results suggest that *Gr33a* could work as a general co-receptor required for detecting bitter molecules. However, misexpression of *Gr66a*, *Gr93a* and *Gr33a* in a cell which normally does not respond to caffeine is not sufficient to allow these cells to respond to this molecule (Moon *et al.*, 2009), which suggests that another element is still missing.

II. The MultiCAFE: a quick feeding preference test to build dose-response curves

1. Feeding preference tests in *D. melanogaster*

In our work, we needed a behavioral assay to underline potential differences of feeding preferences with or without exposure to an antifeedant compound, in order to highlight habituation. Several behavior tests have been developed to assess feeding preferences in the fruit fly. Here, we are describing the most commonly used.

A. Test based on the fly density

The simplest approach consists in recording how many flies wander on a treated surface as compared to a control surface (Marella *et al.*, 2006) (Figure 3). This measure is not directly linked to consumption but relies on the fact that flies use taste receptors of their legs and of their mouthparts to check the substrate on which they stand. A preference index is built by counting the number of flies on the two media at different time intervals: $I = (\text{Nb of flies on Test medium} - \text{Nb of flies on Control medium}) / (\text{Nb of flies on Test medium} + \text{Nb of flies on Control medium})$. An index comprised between 0 and 1 shows an attraction towards the test medium, while an index between -1 and 0 shows a deterrence (0 represents neutrality).

Although this test works well when the aversion or the attraction towards one of the media is high, its sensitivity decreases quickly as the two media get closer in taste (personal observation). Flies may feed more on one of the media but they do not seem to spend more time on the preferred medium in this case, leading to an index biased towards indifference. Thus, the results of this test for fine discrimination cannot be trusted.

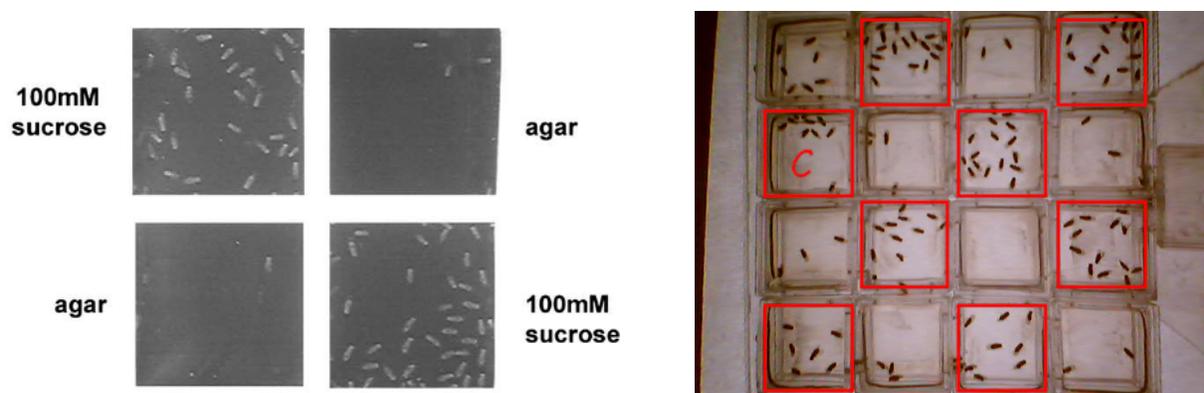


Figure 3. Examples of results from the taste assay based on fly density.

Left: Flies were given the choice between plain agar and agar supplemented with sucrose 100 mM (Marella *et al.*, 2006). **Right:** Flies were given the choice between plain agar (red squares) and agar mixed with sucrose 100 mM and quinine 1 mM (personal results).

B. Proboscis extension reflex (PER)

When the taste sensilla of a hungry fly are in contact with a phagostimulant substrate, the fly extends its proboscis in order to feed on this medium. The PER assay exploits this behavior (Dethier, 1976). This method consists in immobilizing starved flies and stimulating their proboscis or their tarsi with different solutions (Figure 4). In these conditions, the proportion of flies extending their proboscis is high if the solution is appetitive and low if the solution is aversive, and this in a dose-dependent manner. The proportion of flies extending their proboscis for the different molecules tested allows a comparison of their hedonic value. This test does not necessitate much material and has been used extensively on different insects since it was developed in the 1920s (Minnich, 1921). In *D. melanogaster*, PER can be used to measure the response of wild type or mutant flies to sugars (Rodrigues and Siddiqi, 1981; Ishimoto *et al.*, 2000; Nisimura *et al.*, 2005) or sugars mixed with antifeedants (Meunier *et al.*, 2003; Gordon and Scott, 2009) for example. PER can also be associated to rewards (Chabaud *et al.*, 2006) or punishments (Masek and Scott, 2010) in order to create associative learning.

As the previous test, the PER does not rely on consumption but on the detection of the molecules by the taste sensilla. Moreover, the PER is an individual assay and, as such, can require a lot of repetitions for each condition tested.



Figure 4. Proboscis Extension Reflex assay (PER).

Upon stimulation of the tarsi with 100 mM sucrose, the fly extends its proboscis (Gordesky-Gold *et al.*, 2008).

C. Two-choice test using food dyes

The most commonly used test consists in allowing flies to feed in the dark on two food substrates mixed either with a blue or a red food dye (Tanimura *et al.*, 1982) (Figure 5). After exposure to the food, the abdomen color of each fly is checked (red, blue or purple when they fed on both sources, empty when they did not feed) and a preference index is computed. If the tested substance was associated with the blue dye, the index will be: $I = (\text{Nb blue flies} + \frac{1}{2} \text{Nb purple flies}) / (\text{Nb blue flies} + \text{Nb purple flies} + \text{Nb red flies})$. If the index is comprised between 0 and 0.5, the tested molecule is attractive, if the index is between 0.5 and 1, the substance is considered as deterrent. An index of 0.5 shows neutrality.

This test has a good sensitivity and relies on the actual consumption of the flies and not only their presence. Nevertheless, it is limited to the study of binary choices and requires an experienced observer to assess the color of the flies' abdomen. The amount consumed by the flies can be estimated with a spectrophotometer (Tanimura *et al.*, 1982) under the assumption that the content of the flies' abdomen reflects what has been ingested.

As we previously mentioned, the test based on food dyes, which has already been used in many studies and has a good sensitivity, relies on the actual consumption of the flies and not only their presence. Nevertheless, the major drawbacks of this test are its inability to perform

more than two-choice assays and the relative difficulty in assessing the color of the abdomen. The amount consumed by the flies can be estimated but a spectrophotometer is required. Moreover, the consumption of the flies cannot be monitored through time as the flies must be sacrificed in order to get the results of the test.



Figure 5. Colored wells assay.

Left: 96-microwell plate filled with the two agar solutions tested (Isono and Morita, 2010). Right: After the test, the number of flies having a blue, red or purple abdomen is determined.

D. Capillary feeder (CAFE)

In rats and mice, “self-service bottles” are commonly used to study feeding behaviors (Glendinning *et al.*, 2005; Pittman *et al.*, 2006; Inoue *et al.*, 2007; Tordoff *et al.*, 2008). The same principle has been used in insects, such as *ad hoc* capillary feeders for houseflies (Dethier, 1976) or 100 μ l capillaries for the flesh-fly *Sarcophaga bullata* (Cheung and Smith, 1998). More recently, Ja *et al.* (2007) studied the feeding behavior of *D. melanogaster* adults with 5 μ l micro-capillary tubes. With this system, called Capillary Feeder (CAFE), they analyzed the prandial behavior of flies, the influence of population density or humidity and the impact of ethanol or paraquat on food intake (Figure 6). The quantity of liquid ingested by the flies can be recorded in real time by monitoring the level of the liquid within the capillaries. This test has been used successfully as a no choice or two-choice assay on *D. melanogaster* to study the regulation of feeding by peripheral clocks (Xu *et al.*, 2008; Chatterjee *et al.*, 2010), the effect of leucokinin on meal size regulation (Al-Anzi *et al.*, 2010) or how the food content in protein and carbohydrate

affects lifespan and fecundity (Lee *et al.*, 2008) or sleep-wake behavior (Catterson *et al.*, 2010) for example.

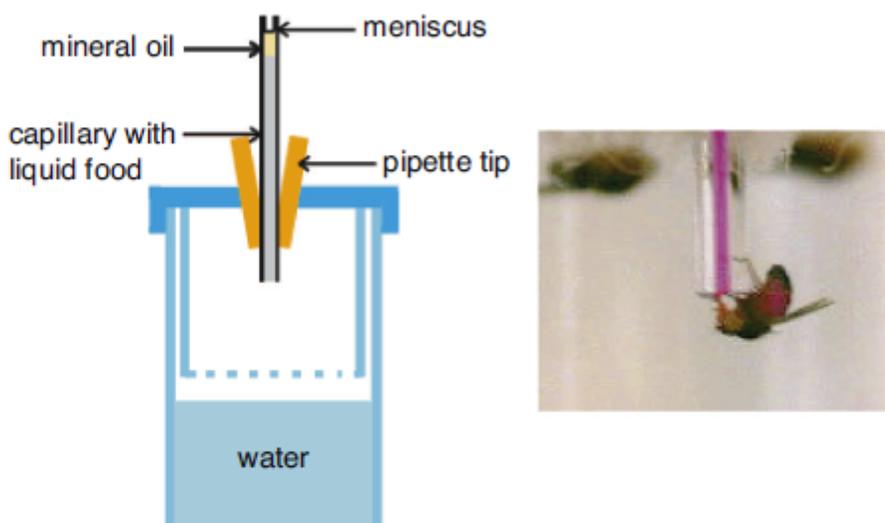


Figure 6. The CAFE assay.

The level of liquid in the capillary tubes is monitored and consumption can be measured through time (Ja *et al.*, 2007).

2. Introduction to a quantitative multiple-choice assay

We have described various behavioral tests available to measure feeding preferences in *D. melanogaster*. All these tests have proven to give results but they also have disadvantages. Most of them are not directly related to the consumption, or the quantitative data are not readily accessible. The CAFE assay seemed to be the only available test to fulfill this condition. Moreover, in order to build dose-response profiles quickly, we chose to develop a multiple-choice test. Given the limitations of existing assays, we tried to design another approach to evaluate flies selectivity and absolute consumption. We adapted the CAFE assay and evaluated the use of a system to test feeding preferences in flies by providing them access to a series of 6 capillary tubes filled with solutions containing different concentrations of an antifeedant. This approach, that we called MultiCAFE, gives the possibility to build dose-response profiles directly.

However, some theoretical problems arise from this setup. One of the potential limitation of the MultiCAFE is that it may not make it easy for flies to discriminate among the different capillary feeders because of the multiplicity of choices available (Prince *et al.*, 2004) (Figure 7). The consumption of two substances or two concentrations can differ greatly whether they are presented alone or simultaneously (Shimada *et al.*, 1987; Akhtar and Isman, 2004). This could influence the apparent antifeedant potency of a given concentration of a bitter substance in the MultiCAFE. Binary choices might be easier to deal with for the flies. Indeed, memorization and comparison of the options should be quicker when only two choices are provided, rather than when many different types of food are available, even if fruit flies seem to be capable of visual learning (Schnaitmann *et al.*, 2010). On the other hand, the multiplicity of options may introduce such complexity that the flies' choice might involve instant decisions, related to hunger and taste detection, more than memory. In this way, we can wonder if multiple-choice tests can be considered as equivalent to multiple no-choice tests. If this is the case or if, at least, the sensitivity of the multiple-choice test is close to the sensitivity of no-choice or two-choice assays, then the MultiCAFE would give the possibility to compare the antifeedant activity of different substances or to describe mutants' impairments.

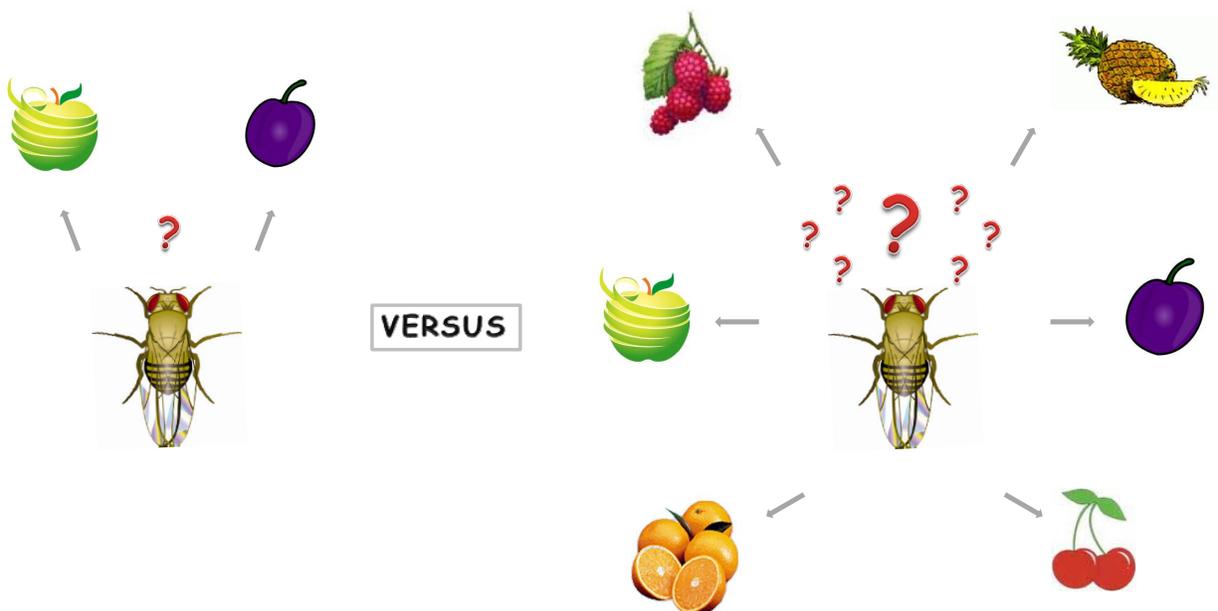


Figure 7. Are multiple-choice preferences more difficult to assess than two-choice preferences in *D. melanogaster*?

The fact that multiple substances (or concentrations) presented at the same time can be more difficult to discriminate, as compared to two-choice assays, might increase the number of repetitions required in order to decrease variability (Raffa *et al.*, 2002). Moreover, we can wonder if providing the flies with both palatable and non-palatable food sources might elicit “compensative” feeding. As the flies do not eat the deterrent food, they may eat more of the appetitive food to compensate and keep a constant total consumption. Some protocol issues may arise as well from multiple-choice assays. Indeed, the way to present the different food sources is likely to have an effect on the choice or the intake of the flies. Moreover, high fly densities might trigger competition for the most palatable food sources.

Using the MultiCAFE, we try to answer some of these questions. First, we show that the fly density as well as the order of presentation of the concentrations has an influence on the quinine dose-response profile, while the spacing between the capillary tubes does not seem to modify the flies’ feeding behavior. Secondly, we evaluate the variability generated by the MultiCAFE according to the number of repetitions. We also compare the sensitivity of the MultiCAFE assay used as a no-choice, two-choice or multiple-choice test. Then, we build dose-response profiles for 8 alkaloids and rank them according to their antifeedant potency in the MultiCAFE. Finally, we test a mutant supposed to have caffeine-detection impairments and show that, in addition to the lower caffeine discrimination, this mutant also seems to have a general intake defect.

3. Description of the MultiCAFE setup

A. First generation of the assay (vials)

Unless otherwise specified, the flies used in these experiments are Canton-S flies, graciously given to our laboratory by Pr. Teiichi Tanimura. Emerged flies (~1 day old) were transferred to a freshly prepared food medium for 2 to 3 days and maintained in a rearing chamber at 25 °C. The flies were first sexed (after numbing them on ice), transferred to plastic tubes provided with humidified filter paper and starved for 20 – 22 hours. Just before the experiment, these flies were numbed on ice and transferred into experimental vials (23.5 dia. ×

40 mm, SARSTEDT). All experiments were performed at the beginning of the afternoon, to prevent any effect of the circadian rhythm, at 25°C under complete darkness.

Experimental vials were closed by a plug (28.5 mm Buzz-Plugs, Fisherbrand), cut to 0.8 cm height and sliced in two halves (Figure 8). On one half of this modified plug, we disposed a row of six 5 µl micro-capillary tubes (Hirschmann Laborgeräte, Germany) on a strip of double-sided sticky tape. The capillaries were equally spaced (~ 1 mm unless otherwise specified) and protruded inside of the vial by ~ 5 mm. Each row of capillary tubes was filled with serial dilutions (0, 0.001, 0.01, 0.1, 1 and 10 mM) of a test compound mixed with 35 mM fructose and 0.125 mg / ml of blue food dye (brilliant blue, FCF (C37H34O9SNa), Tokyo Kasei Co.). According to earlier tests, this dye has no effect on taste sensitivity and is not toxic to flies at the concentration used (Tanimura *et al.*, 1982). As a control, we also tested a row of capillaries with only fructose and the blue dye.

Moreover, as the molecules were presented in solution, evaporation became an issue and the tests had to be conducted at a high humidity rate (~70 %). Limiting evaporation in MultiCAFE experiments is particularly important, for three reasons. First, if one wants to measure consumption accurately, evaporation should be kept to a minimum in order to decrease statistical errors. During the pilot tests, we experienced conditions where evaporation was four or five times higher than the flies' consumption. Reducing evaporation allowed us to reduce variability between tests. Secondly, the controls have to be carefully chosen so that they truly represent the evaporation present in the test tubes. In our dose-response curves, some points are negative, especially at high doses of alkaloids where no ingestion occurs. The most likely explanation is that evaporation in tubes containing flies is reduced as compared to tubes which are empty. Thirdly, evaporation may alter the actual concentration of antifeedants experienced by the flies. Since the liquid column is enclosed into a tube limiting passive diffusion and convection, the surface of the liquid is probably more concentrated in antifeedant (and sugar) than the rest of the tube. So far, the best way to limit this concentration seems to reduce evaporation as much as possible. To limit evaporation, the outer side of each capillary was dipped into mineral oil and the excess of oil was wiped with a paper towel. For each test and for each condition, a control vial without flies was placed into the experimental chamber to monitor evaporation of the capillaries.

The liquid levels in the capillaries were recorded as images with a digital camera or a scanner (HP Scanjet 3770) at 600 d.p.i. before and after the experimental session, and the consumption measured using ImageJ (Abramoff *et al.*, 2004). The actual consumption of the flies was estimated by subtracting the amount of liquid evaporated within the empty vial from this value.

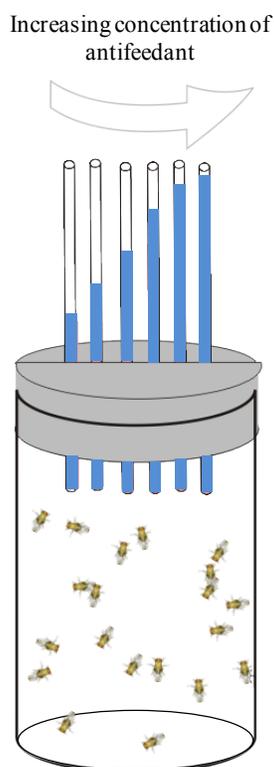


Figure 8. Schematic representation of the first generation of the MultiCAFE assay.

A group of starved flies is inserted into a plastic vial which plug maintains six capillary tubes filled with different solutions. After two hours, the level in the capillary tubes is measured and the consumption of the flies is calculated.

B. Second generation of the assay (boxes)

To further reduce evaporation, we modified the setup, using boxes instead of vials. In this system, the capillaries were inside the box and were consequently less exposed to airstreams (Figure 9). The six capillaries were disposed on a microscope slide with double faced tape and equally spaced (~ 5 mm). The slide was then placed in a plastic box (95 x 76 x 15 mm, Caubère,

France) with repositionable adhesive pads (Patafix, UHU). The flies were transferred into the box without anesthesia.

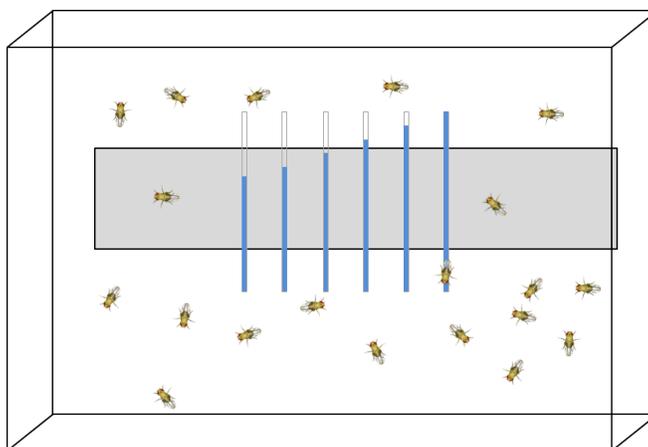


Figure 9. Schematic representation of the second generation of the MultiCAFE assay. The capillary tubes are taped on a microscope slide stuck in the test box.

4. Statistical analysis

The statistical analysis was conducted in collaboration with Pablo Reeb (Universidad Nacional del Comahue, Facultad de Ciencias Agrarias, CC 85 (8303), Cinco Saltos, Argentina).

In no-choice assays, the results obtained for each concentration and each treatment are independent from each other. In this case, unpaired Student's t-tests, ANOVAs or Tukey's tests can generally be used to compare doses or treatments. In the case of two-choice assays, the results obtained for different treatments, for example, are independent and can be treated as in no-choice tests. However, the dependency between the two doses provided to the flies at the same time must be taken into account and the analysis is generally done with paired Student's t-tests.

As we said before, in the MultiCAFE, 6 different doses are provided simultaneously to the flies. The total consumptions, obtained by adding up the consumption in the 6 capillary tubes, can be considered as independent and compared using ANOVAs. However, the consumption in

the 6 tubes present at the same time cannot be considered as independent in any case. Thus, the comparison of the curves cannot involve the aforementioned tests. Multivariate analysis is suitable to the quantitative nature of our response variables (quantity consumed at each concentration) and the dependency among the different factors (identity of the test compound, distance between capillaries, serial or random order, sex) (Roa, 1992; Manly, 1993). To analyze our data, we ran a descriptive multivariate analysis to explore the relationships between variables and then an inferential statistical analysis for the suggested model.

Firstly, we calculated analytically simple linear correlation matrices (Pearson correlation) and we built Scatter Plots Matrices, in order to detect patterns of association of variables and eliminate non-linear correlations that might exist. Secondly, we ran a Principal Components Analysis (PCA) in order to confirm correlations between variables and to study the association with the various classification variables (e.g. substance, sex, series) exploring for possible differences. This analysis is also a way to observe the variability between vials or other classification variables, trying to identify outliers. Scatter Plots Matrices and PCA are exploratory methods which can be interesting to run to get a general idea of the relationships between data.

Then, we studied the assumptions for the implementation of Multivariate Analysis of Variance Models to check the performance of multivariate normality and equality of covariances. Finally, we implemented a Multiple Analysis of Variance (MANOVA, Roy's test unless otherwise specified) to quantify the effect of treatments and compare the treatments of interest. When they resulted significant, profile analyses (Johnson and Wichern, 1998) were used to analyze the patterns of consumption of the groups under study.

5. Influence of fly density on intake in the MultiCAFE

The dose-response curves obtained with the MultiCAFE may combine the taste discrimination capacities of the flies with memory performances (Motosaka *et al.*, 2007) and a number of social interactions like competition (Dierick and Greenspan, 2006; Vrontou *et al.*, 2006) or social facilitation (Shimada *et al.*, 1987; Tinette *et al.*, 2004; Tinette *et al.*, 2007). The

fly density in the test chambers was likely to modulate the agonist and antagonist interactions between animals and thus, to have an influence on the results of the MultiCAFE. In order to establish the impact of the number of flies on MultiCAFE tests, we compared the responses to a series of dilutions of quinine using densities of 10, 20, 40 or 60 flies. Each test condition (density \times sex) was replicated 10 times in vials.

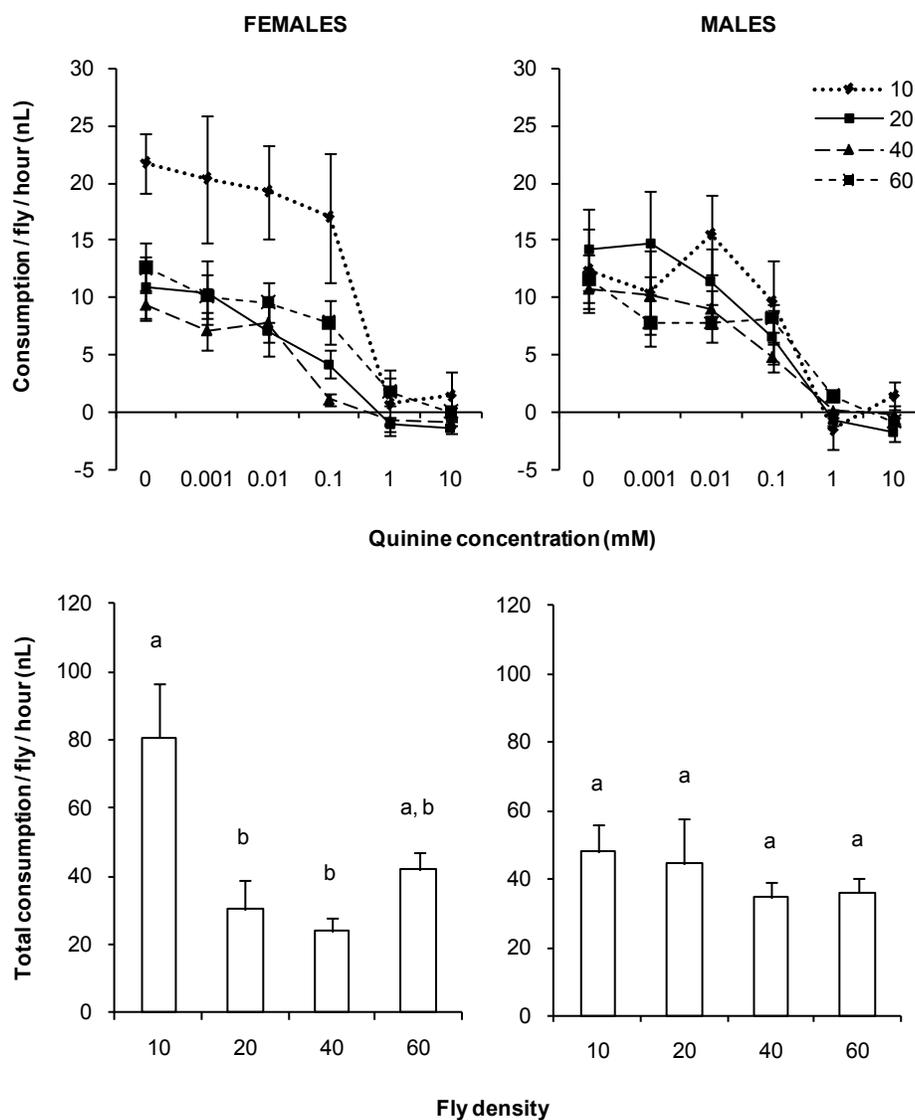


Figure 10. MultiCAFE dose-response curves and total consumption for quinine according to the fly density (10, 20, 40 and 60 flies).

The total consumption was obtained by adding up the consumption in the 6 capillary tubes provided to the flies. $n = 10$ for each curve. Error bars represent S.E.M. On each total consumption graph, data marked by different letters are significantly different (Bonferroni, $p < 0.01$).

There was an effect of the density on the individual consumption both in males ($p = 0.0081$, MANOVA) and in females ($p = 0.0011$, MANOVA) (Figure 10). The females seemed to reduce their uptake with increasing density of the flies in the vial. The dose-response curves looked very similar across all density conditions, with 50% of the inhibition observed between 0.01 and 1 mM quinine and a plateau at 0.001 mM. The major impact of density was observed with 10 flies per tube, with a higher consumption per fly and increased variation across replicates compared to other densities. Conversely, with 60 flies, we observed a lower variability but the dose-response curve seemed to be shifted to the right by about a factor of 10 and showed a slight change in its shape. The results for males were quite similar but the difference of consumption for a density of 10 flies was less marked. The difference observed between males and females for a density of 10 flies is difficult to interpret. A hypothesis would be that the females, more than the males, adapt their feeding strategies according to the population density on the medium. In any case, more data is needed in order to confirm this effect. Given these observations, groups of 20 or 40 flies seem to represent a good compromise between the numbers of replicates required and the total number of flies needed to build a single dose-response curve.

6. Influence of the arrangement of the series of concentration of quinine

Raffa *et al.* (2002) used a multiple-choice assay to test the effect of isopimaric acid on *Lymantria dispar* larvae. They coated leaf disks with various concentrations of isopimaric acid and provided the caterpillars with up to 5 different concentrations at a time. The leaf discs were disposed in circle and the various doses were presented in 6 different configurations. They found that the caterpillars could discriminate between the concentrations of isopimaric acid and that the configuration had an effect on the intake of the larvae. Indeed, the dose response profile had the same shape for each configuration but some arrangements seemed to elicit an increase in the consumption of the lower doses and thus a higher probability to find significant differences of intake between doses.

To assess if the order of presentation of the capillaries had an impact on the dose-response curves in the MultiCAFE, we tested two conditions (a) capillaries disposed in a row of increasing concentrations and (b) capillaries disposed in random order, using groups of 40 flies and 10 replicates per condition and per sex in vials. The 10 randomized order corresponding to

the 10 replicates were obtained using the random function as a macro under Excel. No difference was observed between the curves for males ($p = 0.1843$, MANOVA, Figure 11).

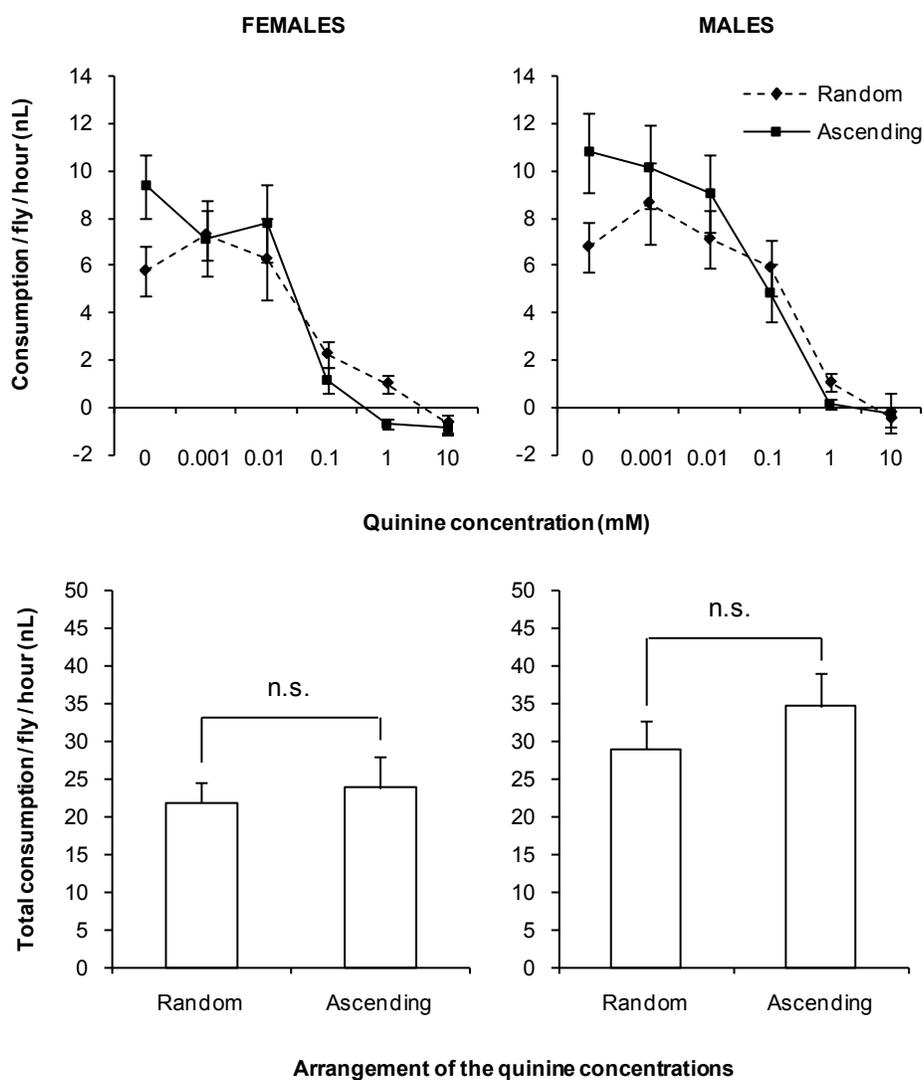


Figure 11. MultiCAFE dose-response curves and total consumption for quinine according to the order of the concentrations tested.

The concentrations were presented either in an ascending or a randomized order. The total consumption was obtained by adding up the consumption in the 6 capillary tubes provided to the flies. $n = 10$ for each curve. Error bars represent S.E.M. For each sex, the total consumption was not significantly different between arrangements of the capillary tubes (ANOVA, $p = 0.6951$ for females and 0.3405 for males).

As in the previous experiment, we found a significant difference in the female consumption according to the arrangement of the series of concentrations ($p = 0.0155$, MANOVA, Figure 11). The two quinine dose response curves looked very alike though the shape of the curves was slightly different. As in the previous experiment, variability increased when the concentration of quinine was low. Since the effect of arranging the concentrations in series or randomly seemed quite modest, we used capillaries arranged in serial order of increasing concentrations in the rest of our experiments.

7. Effect of the spacing of the capillary tubes

When the capillaries were close to each other, we observed that flies could walk from one tube to the other and thus, simultaneously sample different solutions with their legs, which could help discriminate between the solutions provided. On the other hand, if capillaries touch each other, lack of space and competition may happen. In order to assess if the spacing between the multiple food sources affected the responses to quinine, we designed three conditions: capillaries touching each other, or spaced by 1 mm or by 3 mm. Each condition (distance \times sex) was tested 10 times using groups of 40 flies in vials. We did not find any difference between the spacing conditions neither for males ($p = 0.3779$, MANOVA) nor for females ($p = 0.2179$, MANOVA) (Figure 12). In fact, the three dose-response curves obtained were nearly visually identical. Although these observations do not preclude that spacing may affect the results with other antifeedants, we consider this unlikely. Considering these results and for practical reasons, we chose to use a distance of 1 mm between the capillaries in the following experiments.

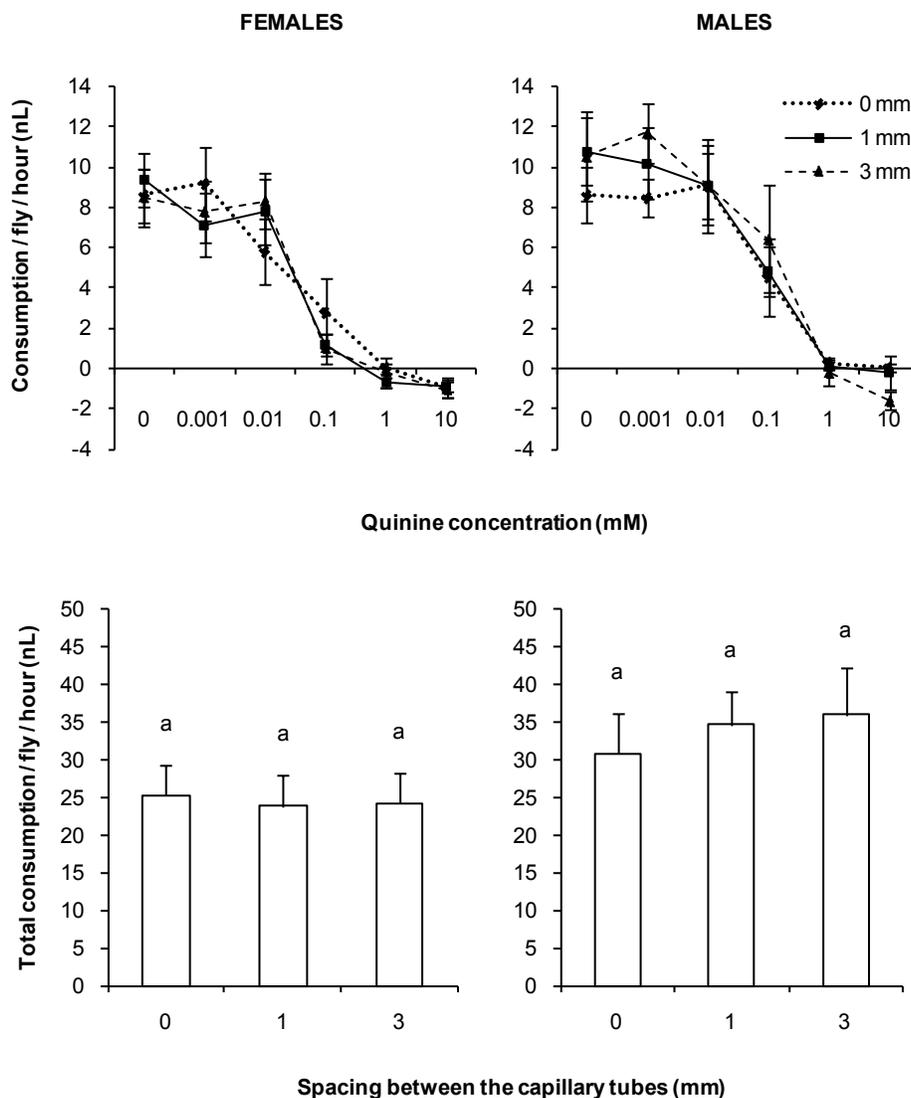


Figure 12. MultiCAFE dose-response curves and total consumption for quinine according to the spacing between the capillary tubes filled with the tested solutions.

The capillaries were either touching each other, spaced by 1 mm or by 3 mm. The total consumption was obtained by adding up the consumption in the 6 capillary tubes provided to the flies. $n = 10$ for each curve. Error bars represent S.E.M. For each sex, the total consumption was not significantly different between spacings (ANOVA, $p = 0.9640$ for females and 0.7865 for males).

8. Number of replicates needed to build a dose response-curve

This first set of data led us to consider that 10 repetitions for each experimental condition could be considered as a reasonable number to get a good estimate of the dose response curves obtained with quinine. In order to go beyond this rule of thumb, we ran a statistical estimate of the reduction of variability obtained when using increasing numbers of repetitions. We used all experiments performed with the fructose control and randomly selected subsets of these data to estimate the variability. As shown on Figure 13, we observed that the standard deviation reached a plateau at about 15 repetitions.

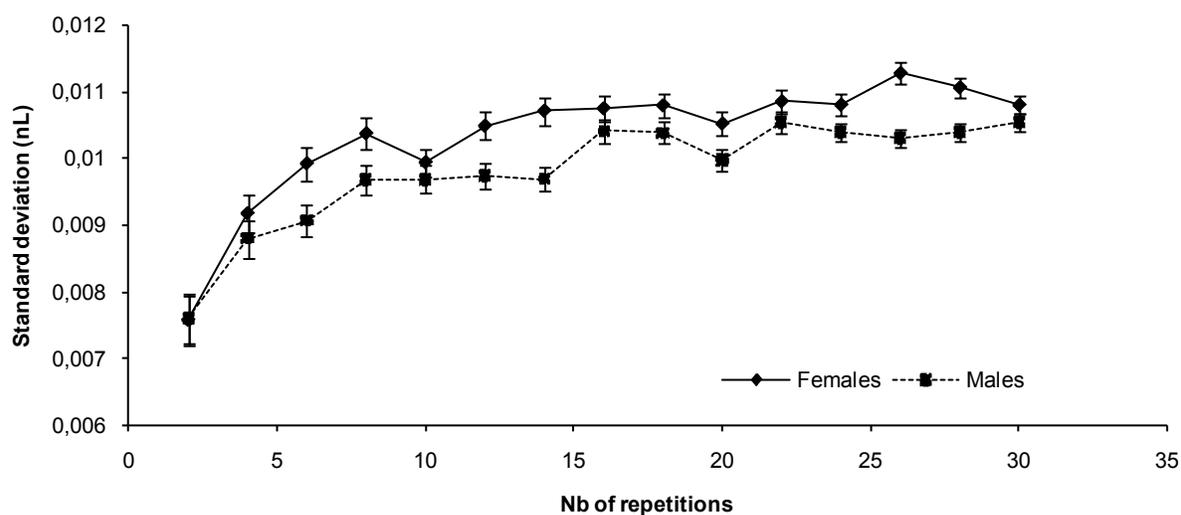


Figure 13. Evolution of the standard deviation of the data obtained with the MultiCAFE assay according to the number of repetitions.

We randomly selected subsets of the experiments performed with the fructose control and statistically estimated the reduction in variability obtained when using increasing numbers of repetitions. Error bars represent S.E.M.

9. Comparison of the test used as a no-choice, two-choice or multiple-choice assay

As we said earlier, the multiplicity of choices might make it more difficult for the flies to discriminate between the different options provided. Thus, multiple-choice assays are likely to be less sensitive than no-choice or two-choice tests. To assess the sensitivity of the MultiCAFE, we

built a dose response curve for quinine using the test as a no-choice or a two-choice assay, in order to compare the results with the multiple-choice curve. For these experiments, we used the second generation of the setup.

In the no choice experiment ($n = 6$, 20 unsexed flies per box), the 6 capillaries contained the blue dye, fructose and one concentration of quinine (0, 0.001, 0.01, 0.1, 1 or 10 mM). In the two – choice test experiment ($n = 10$, 20 unsexed flies per box), all capillaries contained the blue dye and fructose alone and half of them were added with one concentration of quinine (0, 0.001, 0.01, 0.1, 1 or 10 mM). In this case, we expressed the consumption in the capillaries containing quinine as a percentage of the consumption in the capillary containing only fructose. The multiple-choice test ($n = 20$, 20 unsexed flies per box) was conducted as in the vials.

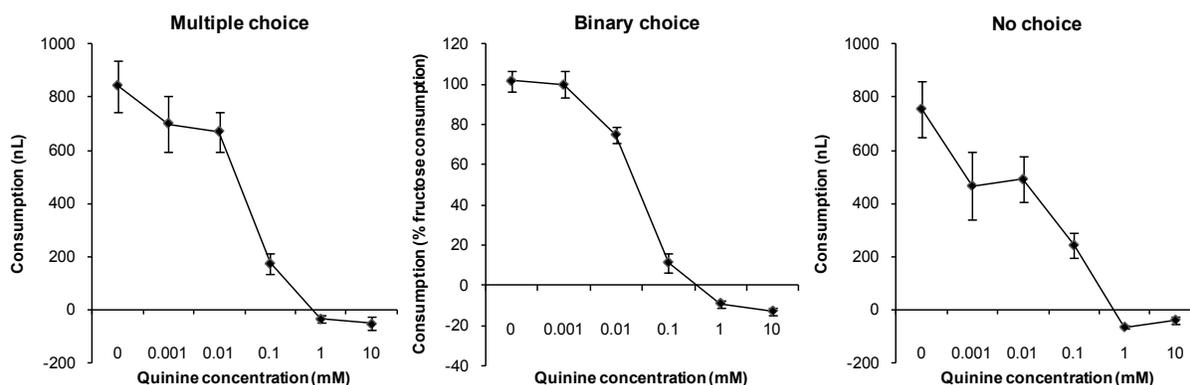


Figure 14. Comparison of quinine dose-response profiles obtained with the MultiCAFE used as a multiple-choice, a binary choice or a no-choice assay.

For the binary test the consumption in the capillaries containing quinine is expressed in percentage of the consumption in the capillary containing only fructose. $n = 20$ for the multiple-choice test, $n = 10$ for each concentration of the binary test and $n = 6$ for each concentration of the no-choice test. Error bars represent S.E.M.

We obtained similar dose-response curves for the three conditions (Figure 14). We estimated graphically the half maximal effective concentration (EC_{50}) of these curves by determining the concentration of quinine eliciting a consumption equal to 50 % of the consumption of fructose alone. The EC_{50} value was very close for the three kinds of experiments and was around 0.02 – 0.03 mM.

As we said earlier, the multiplicity of choices could have been an issue. However, the similarity between the curves obtained with the MultiCAFE used as a no choice, two-choice or multiple-choice assay clearly shows that the sensitivity of the three kinds of experiments do not differ in our conditions as the flies seem to discriminate the concentrations as easily in the multiple-choice setup as in simpler preference tests.

10. Determination of the EC₅₀ of various alkaloids

Alkaloids form one of the most diverse groups of secondary substances in plants, though most of them are derivatives of a quite restricted range of common amino acids. Over 10,000 alkaloids are known and new structures continue to be found. At least 15 % of the vascular plants contain alkaloids in concentrations of more than 0.01% dry weight and this concentration can even increase in case of herbivore damage. They seem to be present in higher concentrations in the inflorescences, the plant buds and the peripheral epidermal cells, in order to repel herbivorous mammals and insects (Bernays and Chapman, 1994; Roberts and Wink, 1998; Schoonhoven *et al.*, 1998). By recording the activity of “bitter-tuned” neurons or monitoring the aversive response elicited by bitter compounds, many studies have highlighted the diversity of organisms sensitive to alkaloids. Among these organisms, we find mammals like rodents (Mueller *et al.*, 2005; Tordoff *et al.*, 2008), humans (Cubero-Castillo and Noble, 2001; Ley, 2008) or other primates (Danilova *et al.*, 1998; Laska *et al.*, 2009), insects such as *Heliothis virescens* (Ramaswamy *et al.*, 1992) or *Bombyx mori* (Asaoka and Shibuya, 1995) but also the frog (Katsuragi *et al.*, 1997), the goldfish (Lamb and Finger, 1995), *Caenorhabditis elegans* (Hilliard *et al.*, 2004) or leeches (Kornreich and Kleinhaus, 1999) for example.

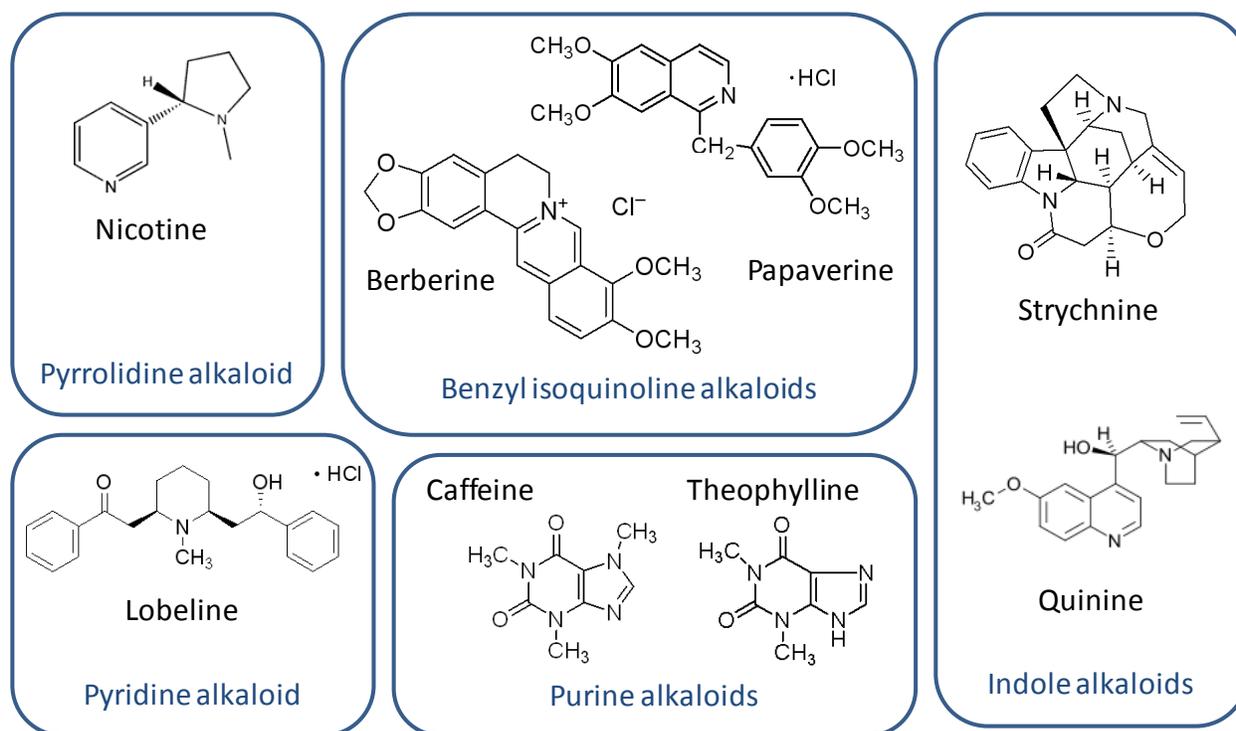


Figure 15. Molecular structure of the alkaloids tested in the MultiCAFE.

We tested eight common alkaloids: berberine, caffeine, lobeline, nicotine, papaverine, quinine, strychnine and theophylline (Figure 15). Each experimental condition was repeated 10 times per sex in vials, using groups of 20 flies. Data from males and females were pooled for these experiments as no significant differences were found between the sexes ($p = 0.4170$ for fructose alone and $p = 0.9815$ for the alkaloids, MANOVA).

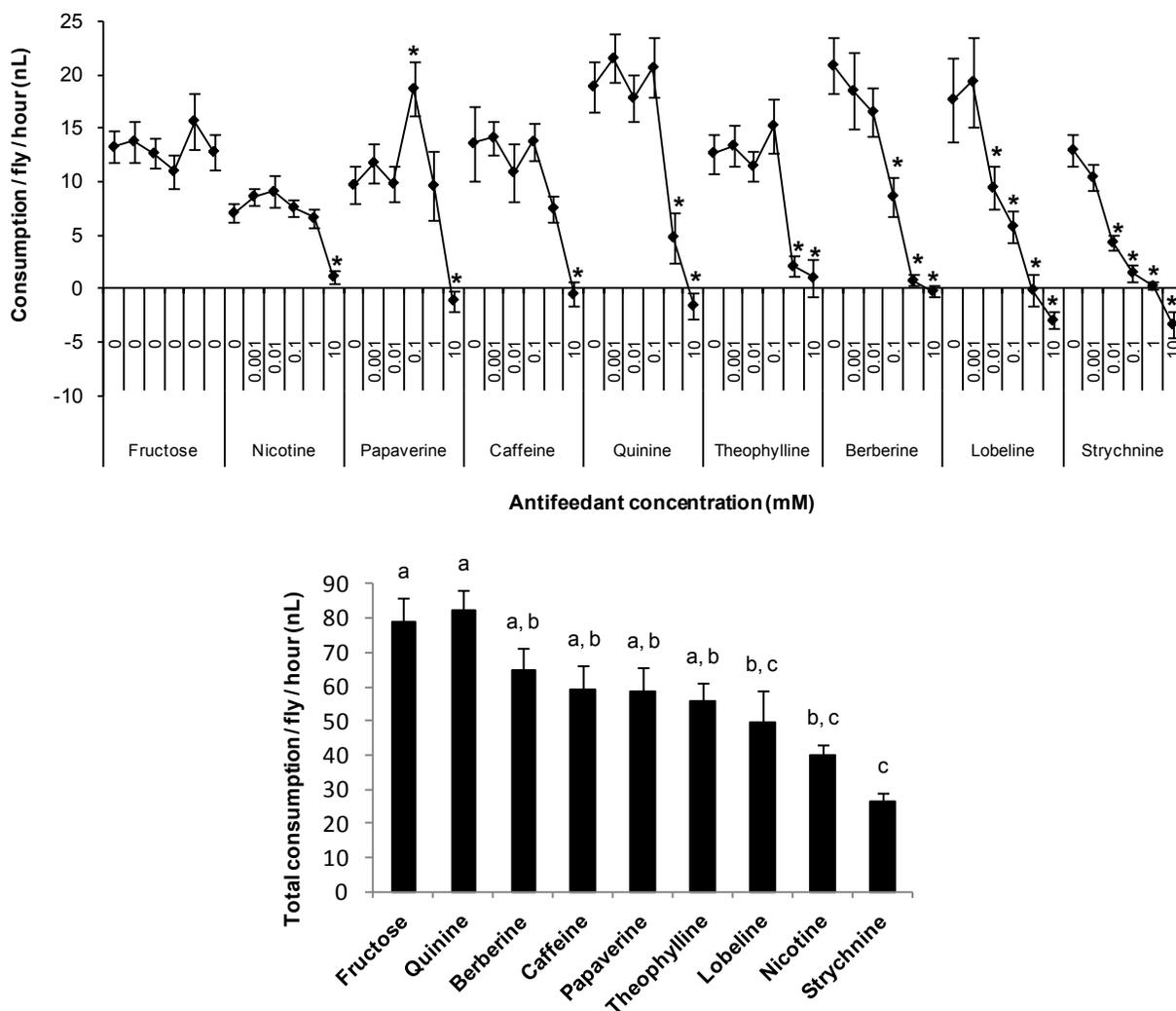


Figure 16. Dose-response curves and total consumption for fructose alone or mixed with various alkaloids. We used different concentrations of nicotine, caffeine, quinine, papaverine, theophylline, lobeline, strychnine and berberine. The fructose response corresponds to a control where the six capillary tubes are filled with the same solution (fructose 35 mM and the blue dye). The total consumption was obtained by adding up the consumption in the 6 capillary tubes provided to the flies. $n = 20$ for each curve. Error bars represent S.E.M. On the curves, the asterisks represent concentrations for which the consumption is significantly different from the intake of fructose alone on the same curve (MANOVA, Profile analysis, $p < 0.01$). For the total consumption, data marked by different letters are significantly different (Bonferroni, $p < 0.05$).

Each of these chemicals was found to inhibit feeding according to the dose (Figure 16). They differed however by their threshold of activity. This activity was estimated graphically by measuring the EC₅₀ from the curves. These values represent the concentration of antifeedant leading to a consumption equal to 50 % of the consumption in the capillary containing fructose only. According to EC₅₀, the biological activity of this series of alkaloids was as follow: strychnine > lobeline > berberine > theophylline > quinine > caffeine > papaverine > nicotine (Table I).

Table I. EC₅₀ of the eight alkaloids tested.

Compound	EC ₅₀ (mM)
Strychnine	0.005
Lobeline	0.011
Berberine	0.06
Theophylline	0.4
Quinine	0.5
Caffeine	1.1
Papaverine	3
Nicotine	4

To our knowledge, this work is the first to examine the bitter potencies of these ten alkaloids in the same strain of flies. Consequently, it is difficult to compare the bitterness ranking obtained here with other studies. However, our ranking is consistent with what has been found on *D. melanogaster* (Meunier *et al.*, 2003; Ueno *et al.*, 2006) and other insect species (Dethier and Bowdan, 1989; 1992; Shields *et al.*, 2008). If we compile the results obtained in the aforementioned studies, we obtain the following ranking: berberine > quinine > strychnine > caffeine > nicotine. This is very similar to what we find except for strychnine which seems to be more potent in our tests.

One of the questions we had concerned compensative feeding in multiple choice assays. If we look at the total consumption for each substance, we can see that compensative feeding did not happen for all the substances (Figure 16). The total consumption of quinine or berberine was equal to the consumption of fructose alone. This shows that the flies compensated the low intake in the capillary tubes containing high concentrations of antifeedants by feeding more in the tubes containing low concentrations. This was also the case for caffeine, papaverine and theophylline to some extent. Indeed, despite the fact that the flies seemed to compensate a little less than for quinine and berberine, the total consumption for these substances was not significantly different from the fructose consumption. However, the flies behaved differently for lobeline, nicotine and strychnine, for which there was no compensative feeding. We can advance some hypotheses.

First these substances may have toxic effects on the flies which could decrease their general intake. A second explanation would be that these molecules damaged the sensilla and the gustatory receptor neurons.

11. Response of a $\Delta Gr66a$ mutant to caffeine with the MultiCAFE

The $\Delta Gr66a$ strain has been reported to be deficient in caffeine detection with the colored wells assay (Moon *et al.*, 2006). In order to observe the behavior of these flies in the MultiCAFE, we compared their response to caffeine and fructose with the response of w^{1118} flies, as the $\Delta Gr66a$ strain was made from a w^{1118} background. This experiment was conducted with the second generation of the MultiCAFE assay ($n = 10$ for each curve). We used only males as preliminary experiments showed that in w^{1118} flies, males had a higher consumption of caffeine than females.

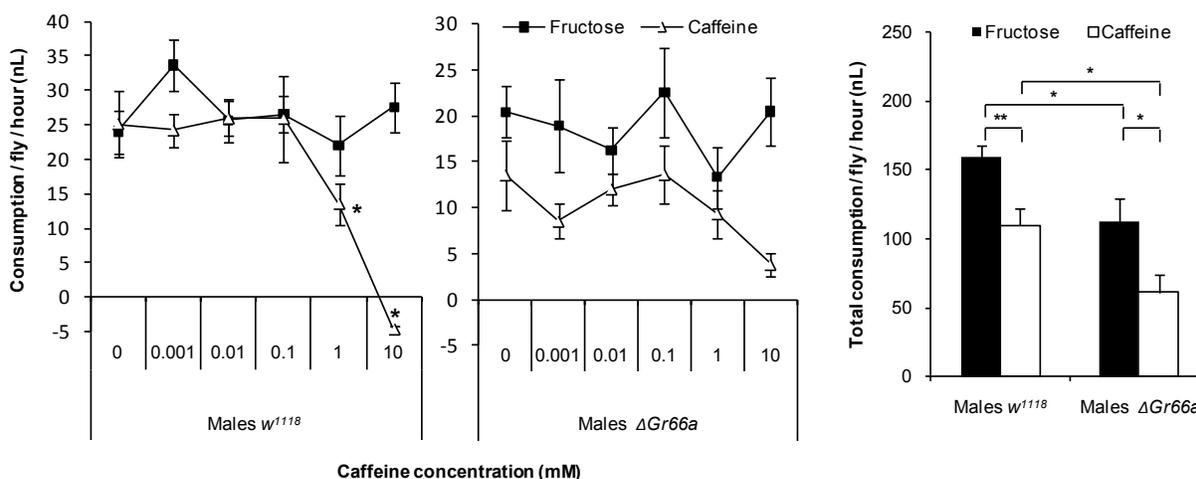


Figure 17. Dose-response curve and total consumption for caffeine and the fructose control, tested on $\Delta Gr66a$ and w^{1118} males.

Only males were tested, as preliminary results showed that males of w^{1118} background had a higher consumption than females. The total consumption was obtained by adding up the consumption in the 6 capillary tubes provided to the flies. $n = 10$ for each curve. Error bars represent S.E.M. On the curves, the asterisks represent concentrations for which the consumption is significantly different from the intake of fructose alone on the same curve (MANOVA, Profile analysis, $p < 0.01$). Differences between total consumptions were calculated using ANOVAs (*: $p < 0.05$, **: $p < 0.01$).

We found that $\Delta Gr66a$ flies consumed less overall than w^{1118} flies (Figure 17, $p = 0.0009$, ANOVA). The total consumption of fructose mixed with caffeine was lower than the total consumption of fructose alone ($p = 0.0006$, ANOVA). This suggests that $\Delta Gr66a$ mutants are not only affected in the detection of caffeine, but they may also detect sugars with less intensity or react differently to starvation than w^{1118} flies. .

The curves for fructose were significantly different between the two strains, which seemed to confirm the fructose detection deficiencies or hunger defects in $\Delta Gr66a$ flies ($p < 0.05$, Hotelling). We then compared the different caffeine concentrations to the capillary tube containing fructose alone. The w^{1118} strain showed a clear caffeine dose-response curve with a good discrimination of caffeine at high concentrations, the highest doses tested being different from the caffeine-free capillary tube ($p < 0.01$, MANOVA using the Bonferroni criterion). On the other hand, the caffeine dose-response curve for $\Delta Gr66a$ flies is much flatter and there is no difference between the concentrations. We did not find any difference between concentrations in the two fructose dose-response curves.

Our results on $\Delta Gr66a$ flies confirm that *Gr66a* is involved in caffeine detection. Indeed, the flies lacking *Gr66a* have trouble discriminating the different concentrations of caffeine. However, unlike the tests used in other studies which rely on relative consumption indexes, we were able to detect with MultiCAFE that $\Delta Gr66a$ flies consume less than w^{1118} control flies. We suspect that $\Delta Gr66a$ flies may have a hunger deficiency which decreases their uptake whatever the substance. Three hypotheses may arise from this statement. First, the two genes flanking *Gr66a* might be involved in uptake regulation. Indeed, the $\Delta Gr66a$ mutant was obtained by the excision of this gene, an excision that also disrupted the two flanking genes, CG7066 and CG7188 (Moon *et al.*, 2006). Secondly, the deletion of *Gr66a* itself could provoke a decrease in consumption. It would be interesting to see if similar situations exist by testing other strains with a deletion of a gustatory receptor gene, like $\Delta Gr93a$ or $\Delta Gr33a$ for example. Thirdly, this strain might react differently to the rearing conditions. Indeed, at the time we did the experiments, these flies were reared at 22°C. Later, we observed that the vigor of the strain improved at 25°C.

12. Conclusion on the MultiCAFE

In this work, we introduced a new behavioral test to evaluate the feeding responses of flies to water-soluble chemicals mixed within a sugar solution. This approach, adapted from the CAFE assay (Ja *et al.*, 2007), gives the possibility to build dose-response curves and to screen for the bioactivity of molecules quickly. Several questions arose from the multiplicity of choices provided to the flies and we tried to answer some of them.

First, we assessed the robustness of this approach by comparing dose-response curves for quinine obtained in different experimental conditions (flies density, serial or random order of the capillaries, spacing between capillary feeders). As we thought, the number of flies present in the assay had a slight influence on the results of the MultiCAFE. We ran the experiment with quinine using 10, 20, 40 or 60 flies. When tested in groups of 10, the flies ate significantly more but we did not observe any marked differences between the higher density conditions. Moreover, the shape of the curve, and thus the choices made by the flies, was very similar at the four densities tested. This lack of density effect is consistent with previous work showing that the choice of a single fly alone is very similar to the choice of a group of flies (Shimada *et al.*, 1987). Even if social interactions are likely to happen during the test, they may have an effect on the flies' intake but they do not seem to play a decisive role in feeding choices, under the present conditions. The arrangement of the different concentrations of quinine also had an effect on the feeding behavior of the flies. Nevertheless, this effect looked quite low and flies did not have trouble discriminating between the concentrations of quinine, whether they were presented in a random or ascending order. In opposition to the fly density and the arrangement of the concentrations, the spacing between the capillary tubes did not influence the flies' intake and discrimination. This suggests that the flies could have access to the capillary tubes in each situation and that they were able to process each concentration, even when the tubes were too far apart to allow the flies to be in contact with several at the same time.

In a second step we showed that the number of repetitions required to make the variability acceptable was around 15. This may be higher than for no-choice or two-choice assays, which elicit lower variability. On the other hand, in these kind of tests, as every concentration has to be tested separately, the number of repetitions required must be multiplied by the number of concentrations tested, which in total gives a higher number of repetitions than

for the MultiCAFE. As we said previously, the multiplicity of choices available could be one of the limitations of the MultiCAFE, making it more difficult for the flies to discriminate among the capillary tubes. However, we obtained very similar curves and EC₅₀ with the MultiCAFE used as a no choice, two-choice or multiple-choice assay which suggests that the sensitivity of the three kinds of experiments may not be so different in our conditions.

Then, we were able to evaluate the activity of eight alkaloids using the MultiCAFE to build the corresponding dose-response curves. We ranked these molecules according to their antifeedant potencies and obtained a bitter ranking consistent with the partial data previously found on these compounds. Moreover, we tested with the MultiCAFE a strain previously reported to have deficiencies in caffeine detection (Moon *et al.*, 2006). We showed that, in addition to the deficiencies in caffeine detection, this mutant had a lower general intake.

The MultiCAFE presents a number of advantages over existing feeding choice. It gives quantitative results that are directly readable, in contrast to the colored wells test for which a spectrophotometer is required to measure how much food was consumed. Such measures are valid only if flies did not empty their crop during the period of observation through defecation or regurgitation. Highlighting a general difference in consumption between $\Delta Gr66a$ and w^{1118} flies was made possible in the MultiCAFE because it is a quantitative test and not a test based on indexes. MultiCAFE is also much less fly and chemical-consuming: in order to build a dose-response curve with 6 concentrations, MultiCAFE experiments require only half the number of flies and nine times less chemicals than the colored wells test (Table II).

Table II. Comparison of the need in flies and substance volume between the MultiCAFE used as a multiple-choice test and the test of the colored wells, in the case of a dose-response curve of six concentrations.

	Need in flies and solution volume for a dose-response curve of 6 concentrations
MultiCAFE	20 repetitions x 20 flies = 400 flies 20 repetitions x 5 μ L per capillary = 100 μ L per concentration
Wells test	3 repetitions x 50 flies x 6 concentrations = 900 flies 3 repetitions x 30 wells x 10 μ L per well = 900 μ L per concentration

The relative consumptions per capillary are not independent from one another and correspond to multiple comparisons between concentrations. This makes it more difficult to analyze the data statistically (Peterson and Renaud, 1989; Roa, 1992; Manly, 1993). The MultiCAFE is a way to compare not only the tested concentrations with a control one, but also those tested concentrations between each others. This interdependency has to be taken into account when running statistical analyses on results from this test. The approach outlined in this work takes into account these concerns.

In brief, the MultiCAFE has some issues which still remain to be improved but it is a very interesting test, quite quick and which consumes less flies and chemicals than most other behavioral assays designed to test feeding preferences. It represents a real contribution to the tools available in our laboratory and is currently used by several other members of our team. It also opens to new questions about how the flies are able to discriminate between the different options available. Indeed, we can wonder if the flies use some aggregation pheromone, like 11-*cis*-vaccenyl acetate (Bartelt *et al.*, 1985; Xu *et al.*, 2005), to mark the most palatable food sources or if they emit stress-elicited molecules to warn the other flies about the deterrent solutions. Preliminary results showed that even the number of visits to the capillary tubes seemed to be correlated with the palatability of the solution, which suggests that some kind of memorization or marking is involved.

13. Screening of some molecules extracted from endemic plants of the Canary Islands

We applied our system to evaluate the antifeedant activity of new chemicals on the fruit fly. This study was done in the frame of a collaboration with Azucena Gonzalez Coloma and Adriana Gonzalez Portero (Instituto de Ciencias Medioambientales, CSIC, Serrano 117, 28006-Madrid, Spain / Instituto de Productos Naturales y Agrobiología, PO Box 195, La Laguna, 38206-Tenerife, Canary Islands, Spain).

Azucena Gonzalez Coloma's research aims at finding natural compounds with agronomical interest and applications as biopesticides. She is particularly interested in the compounds present in endemic plants of the Canary Islands. About 27 % of the approximately

1000 native vascular plant species of the Canary Islands are endemic (Juan *et al.*, 2000) and among them, 70 % are endemic of only one of the 7 islands of the archipelago (Carine and Schaefer, 2010). Some of the compounds produced by these plants seem to have a deterrent or a toxic effect on insects (Gonzalez-Coloma *et al.*, 1999; Fraga *et al.*, 2001; Dominguez *et al.*, 2008) and their bioactivity makes them good candidates to be used as biopesticides. Adriana Gonzalez Portero is doing her PhD partly in the laboratory of Azucena Gonzalez-Coloma and partly in another laboratory situated in Tenerife. She used the MultiCAFE to assess the effect on *D. melanogaster* of some molecules (euparine, euparone, pericallone and 6-hydroxytremetone) extracted from *Pericallis echinata* (Asteracea), an endemic plant of the Canary Islands. These compounds are currently tested on caterpillars and aphids in Azucena Gonzalez-Coloma's laboratory. If some of them had a deterrent effect of drosophila then the multiple tools available in the fruit fly would help understand the modes of action of these molecules.

A. Identification of pericallone as a potential deterrent molecule

The main difficulty in this study was the non-solubility of the tested compounds in pure water. This implies to use organic solvents to dissolve them first and then to dissolve this solution in water. There are three conditions to fill: 1) the tested molecule must be soluble in the solvent, 2) the solvent must be soluble in water and 3) the final concentration of solvent in the water solution should be low enough not to be toxic or deterrent for the flies. Preliminary assays were done in order to find the appropriate solvent and concentrations. Unfortunately, euparone could not be tested because neither of the solvents used for this compound was soluble in water.

The MultiCAFE was set up as described earlier. Briefly, 1-2 day old unsexed flies were starved for 20-22 h and then inserted in the test boxes in groups of 25. Each box contained 4 capillary tubes filled with sucrose 50 mM and different concentrations of the tested product (0, 0.5, 0.25 and 0.125 mg/ml). Boxes containing 4 capillaries filled with sucrose 50 mM and 2.5 % (concentration used for 6-hydroxytremetone) or 5% ethanol (concentration used for euparine and pericallone) served as control. The results showed that the deterrent activity of these products on *D. melanogaster* was quite low (Figure 18). Flies did not detect euparine ($p = 7177$, MANOVA). They were sensitive to 6-hydroxytremetone but this molecule seemed to be slightly appetitive (p

= 0.0259). Pericallone was the only molecule which had an aversive effect on this insect ($p < 0.0001$). However, the aversion was not complete. Higher doses might have a clearer effect on the flies but the solubility of these molecules in water may be a limiting factor.

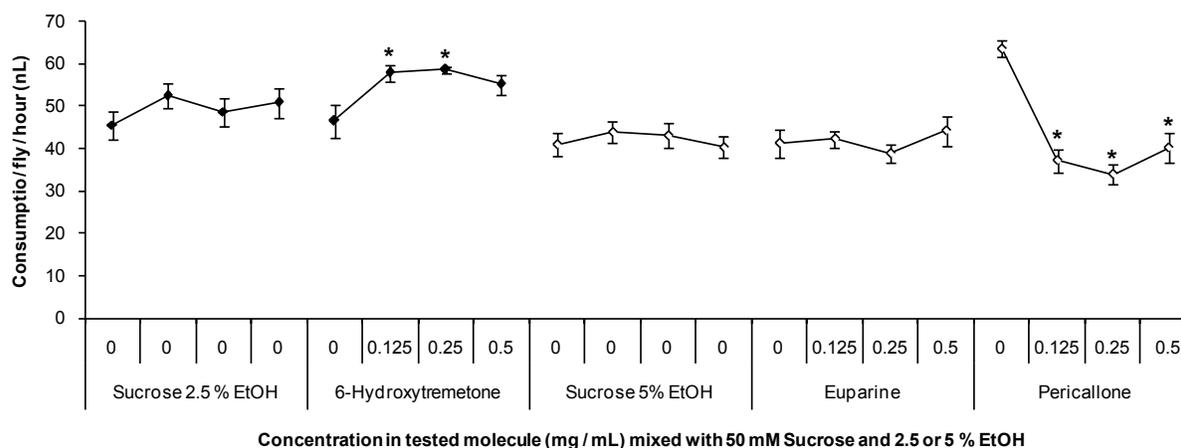


Figure 18. Dose-response curves obtained for sucrose 50 mM mixed with 3 compounds extracted from endemic plants of the Canary Islands: 6-hydroxytremetone, euparine and pericallone.

The curves for sucrose 50 mM with 2.5 or 5 % ethanol were used as control. $n = 22-24$ for each curve. Error bars represent S.E.M. On the curves, the asterisks represent concentrations for which the consumption is significantly different from the intake of fructose alone on the same curve (MANOVA, Profile analysis, $p < 0.05$).

B. Possible inhibitory effect of pericallone on sugar detection

We tested if the antifeedant effect of pericallone we observe with the MultiCAFE could also be found at the peripheral level of the taste system. To this end, the electrophysiological response of some L- and I-type sensilla of the proboscis was recorded using the “tip recording” method (see chapter III.2 for details on the method). The sensilla were stimulated first with sucrose 50 mM, then sucrose 50 mM mixed with pericallone (0.5 mg / ml) and finally, sucrose 50 mM alone again to check for any potential damage on the sensillum. Only the sensilla responding to this last stimulation were included in the analysis. As this study was conducted at the end of Adriana Gonzalez Portero’s stay in our laboratory, only 4 flies could be tested. Although more recordings are needed to confirm the first results, it seemed that pericallone had an inhibitory effect on the sugar cell, which fired less spikes in presence of this compound than for sucrose alone (Figure 19). In some L-type sensilla, the stimulation with sucrose mixed with pericallone elicited two types of spikes, one probably coming from the S-cell and the other type

from the L2 cell. This result is confusing as L-type sensilla have never been described as responding to antifeedants. As we said before, we need more data to be sure of these observations and pericallone has also to be tested alone, without sucrose, to determine if it is detected by the bitter cell.

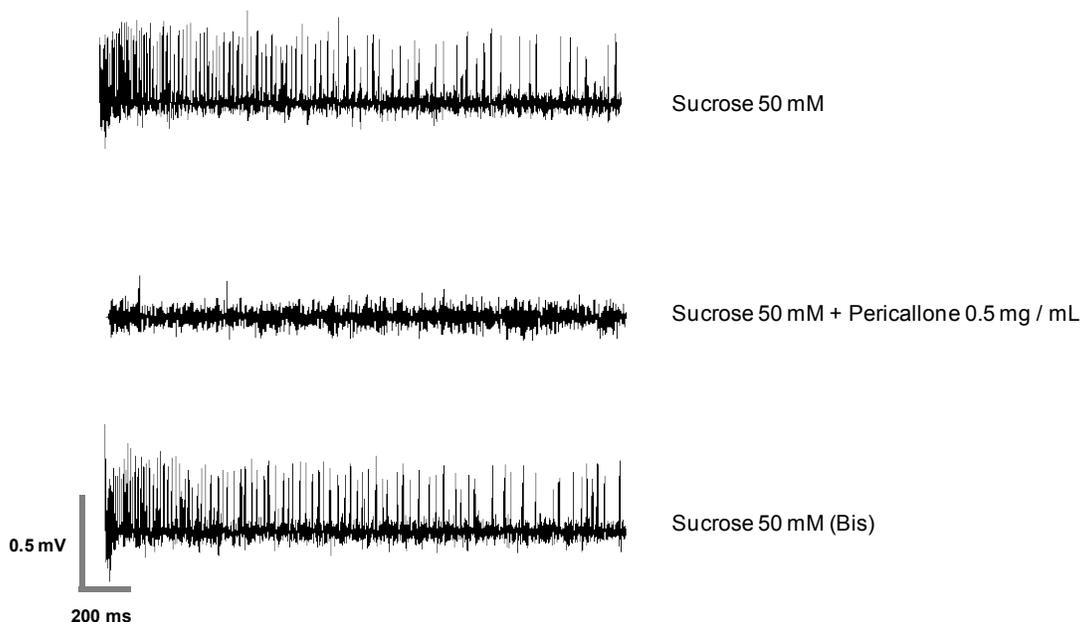


Figure 19. Electrophysiological recordings on a L-type sensillum for sucrose or sucrose mixed with pericallone.

The last stimulation with sucrose was done to check for potential damage on the sensillum.

C. Perspectives of this study

Among the tested products, only pericallone seemed to have a deterrent effect on drosophila and this aversion could come from an effect of this molecule on the peripheral taste system of the flies. This preliminary study was the first step of a collaboration between Azucena Gonzalez Coloma's laboratory and ours. Adriana Gonzalez Portero has applied for a grant to come back for a longer stay in order to test other products, to improve the technique for non-water soluble molecules and to perform more electrophysiological recordings. The problem of the solubility of some molecules in water represents a drawback to the MultiCAFE but we can consider potential solutions. One of them, for example, would be to fill the capillary tubes with sugar, dip them in the solvent containing the molecule and leave them to dry. This method has to be tested but it looks promising

III. Mixture interactions: involvement of the bitter cell in the sugar cell inhibition

1. Introduction

In the first part of this work, we have shown that mixing a sugar with increasing concentrations of antifeedants caused a decrease in the flies' intake. Here we wanted to see if these behavioral observations could be explained, at least partially, by the peripheral taste response of the gustatory neurons. Antifeedants can have two non-exclusive modes of action on insects' taste. As we said earlier, they can elicit a response from the deterrent cells present in the taste sensilla. Many if not all insects possess bitter-sensing cells responding to a subset of antifeedant molecules (Chapman *et al.*, 1991; Glendinning and Hills, 1997; van Loon and Schoonhoven, 1999; Schoonhoven and van Loon, 2002). In *D. melanogaster*, bitter substances seem to be detected by the L2 present in the sensilla of the proboscis (Hiroi *et al.*, 2004; Lacaille *et al.*, 2007), the tarsi (Meunier *et al.*, 2003) and maybe the ovipositor (Mery and Kawecki, 2002; Yang *et al.*, 2008).

Besides activating the deterrent cells, some antifeedants and especially alkaloids have an inhibiting effect on the S cells when presented in mixture with a sugar. This inhibition phenomenon was mentioned fifty years ago (Morita and Yamashita, 1959). It was described more precisely twenty years ago on *Phormia regina* (Dethier and Bowdan, 1989; 1992) and more recently on *Protophormia terraenovae* (Liscia and Solari, 2000) and on the tarsi of *D. melanogaster* (Meunier *et al.*, 2003). For most, if not all, behavioral assays used to study aversive compounds, the tested molecule has to be mixed with sugar to increase the flies' motivation. Nevertheless, mixing the deterrent molecule with a sugar prevents us from knowing if the observed effects come from the detection of the compound by the bitter-sensing receptor neuron or from the inhibition of sugar detection due to this deterrent compound. Considering this fact, understanding how mixture interactions between sugars and antifeedants work would help us distinguish these two modes of action. However, the sugar inhibiting effect of the antifeedants has been mostly neglected and the mechanisms underlying mixture interaction are still unknown.

Several non-exclusive hypotheses can be put forward. First, the aversive and sweet molecules could form a complex in the sensillar lymph, in such a way that the probability of activation of the sugar receptor (and potentially the bitter receptor as well) would decrease. This

could lead to a dose-dependent reduction of the spike frequency. This mechanism is not very likely, though, considering that sucrose detection is inhibited by quinine (Meunier *et al.*, 2003) but that sucrose and quinine don't seem to react together (Nakamura *et al.*, 2002). Secondly, the deterrent molecules could bind to the sugar cells, whether on the same site as the sugar molecules (competitive inhibition), or more probably on a different site. This mechanism has been invoked to explain observations in *Leptinotarsa decemlineata* (Mitchell, 1987). However, Dethier and Bowdan (1989) excluded competitive, non-competitive and uncompetitive mechanisms to explain the sugar detection inhibition in *Phormia regina*. One hypothesis comes from the chemical properties of the compounds: some of the aversive molecules inhibiting sugar detection are amphiphilic, like quinine for example (Naim *et al.*, 1994; Peri *et al.*, 2000). These compounds might permeate through the membranes (at least partly) and interfere directly with transduction cascades for instance. This hypothesis is not to be excluded, although the kinetics of this phenomenon make it unlikely to be the major short-term mechanism.

Another explanation would be a direct interaction between the chemoreceptor cells, especially the deterrent and the sugar cells (Figure 20). An argument in favor of these cell to cell interactions would be the existence of such an interaction in *Schistocerca americana* (White *et al.*, 1990).

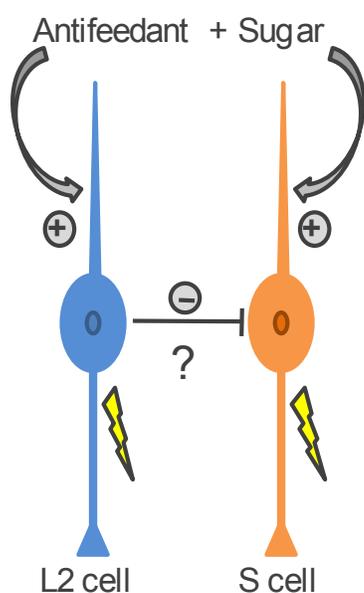


Figure 20. Schematic representation of the potential lateral interaction between the L2 and S cells involved in sugar perception inhibition by antifeedants.

In this work, we show that quinine inhibits fructose detection in the labellar sensilla of the fruit fly. We highlight the correlation between the behavioral data we obtained with the MultiCAFE and this electrophysiological phenomenon. We determine that deterrent molecules inhibit the phasic part of the sugar response as well as the tonic part, which suggest an immediate effect of the antifeedant on sugar detection. Then, we show that the inhibition potency depends on the molecule and that, at the same concentration, some substances inhibit sugar detection almost completely while others do not seem to have any effect. Finally, we test the hypothesis of a lateral interaction between the bitter- and the sugar-sensing cells to explain this inhibition. Using the UAS-Gal4 system, we kill selectively the L2 cell in sensilla sensitive to deterrents and we show that sugar inhibition is not due to an interaction between the L2 and the S cells.

2. Electrophysiological recording technique

The technique used here is called “tip recording” (Hodgson *et al.*, 1955). Flies of 1 – 2 days old were secured to a support with tape and electrically grounded via a glass capillary filled with Ringer’s solution inserted into the abdomen (Figure 21). Individual taste sensilla were stimulated by covering their tip with an electrode containing an electrolyte (1 mM KCl) and a stimulus during 2 sec. To avoid adaptation, consecutive stimulations were applied at least 1 min apart. The recording electrode was connected to a preamplifier compensative for DC offset (gain = $\times 10$, TastePROBE, DTP-02, Syntech, Germany) (Marion-Poll and van der Pers, 1996). Electrical signals were further amplified and filtered by a second amplifier (CyberAmp 320, Axon Instrument, Inc., USA, gain = $\times 100$, 8th order Bessel pass-band filter = 1–2800 Hz). These signals were then digitized (DT9803, Data Translation, USA, sampling rate = 10 kHz, 16 bits), stored on computer and analyzed using dbWave (Marion-Poll, 1996). Spikes were detected and analyzed using software interactive procedures of custom software dbWave. Unless otherwise indicated, we evaluated the action-potential frequency by counting spikes during the first second of recording.

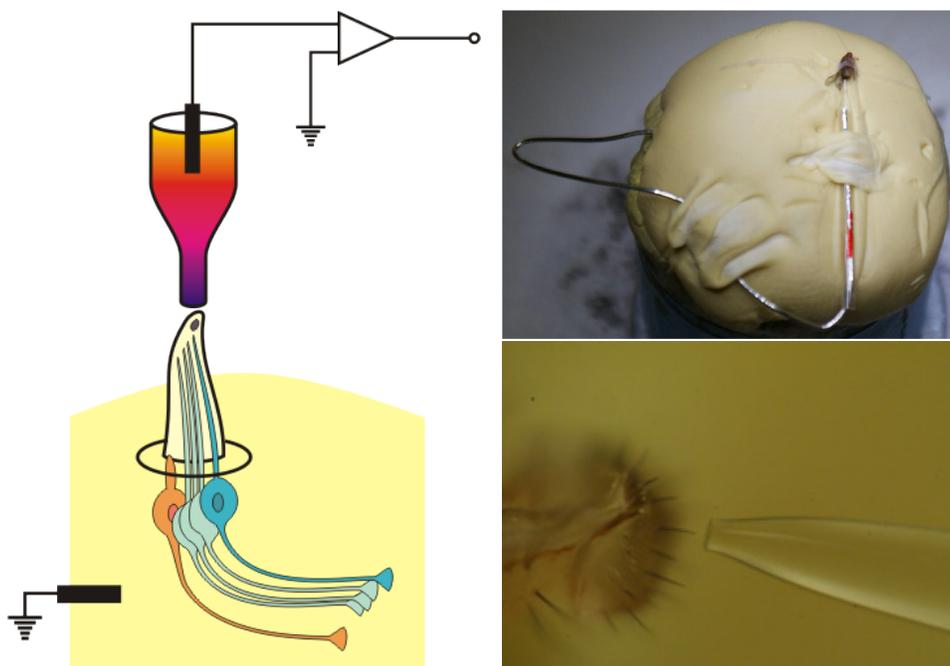


Figure 21. Tip recording method and insect preparation.

Left: Organization of a taste sensillum and principle of the “tip recording” method. **Right:** Electrophysiological preparation. The fly is immobilized on a magnetic support and a reference electrode is inserted in its abdomen. Then, a single sensillum is stimulated with a glass capillary filled with an electrolyte and the stimulus and mounted on a recording silver electrode.

3. Correlation between the electrophysiological and the behavioral responses

We wanted to compare the dose-response curves obtained with the MultiCAFE and the sensitivity of the peripheral receptors as measured with electrophysiology. In order to evaluate the correlation between the MultiCAFE dose-response curves and the sensory responses of the flies taste receptors, we stimulated proboscis sensilla with mixtures of 35 mM fructose and quinine as in the behavioral tests (but without the blue dye). These solutions were tested on two taste hairs of the proboscis, namely I9 and L5 sensilla (Hiroi *et al.*, 2002). I9 sensilla house one neuron sensitive to sugars and one neuron sensitive to bitter compounds while L5 sensilla house four neurons, none of which respond to the bitter substances (Hiroi *et al.*, 2004).

In both sensilla, the total number of spikes recorded during the first second of stimulation decreased as the concentration of quinine increased (Figure 22). This spiking inhibition induced by quinine was fully reversible since we tested fructose alone at the end of the test series and

obtained a comparable level of spikes as at the beginning of the experiment. We further plotted post-stimulus histograms of the responses using 100 ms bins (Figure 23). These data showed that quinine inhibited both the phasic part of the responses (first 200 ms) as well as the tonic responses (after 400 ms). The effect of quinine on fructose detection seemed to be immediate, in opposition to what Meunier *et al.* (2003) found on the tarsi using sucrose. This suggests that the action of antifeedants on sugar detection might depend on the localization of the sensilla ^{and/or} on the sugar involved. Unexpectedly, we did not record a clear increase of firing at high doses of quinine in I9 sensilla as should be expected since one of its cell responds to bitter substances (Hiroi *et al.*, 2004). Further observations are necessary to obtain a set of recordings in which the spikes can be sorted to establish the respective contribution of the sugar- and bitter-sensing neurons to the responses observed.

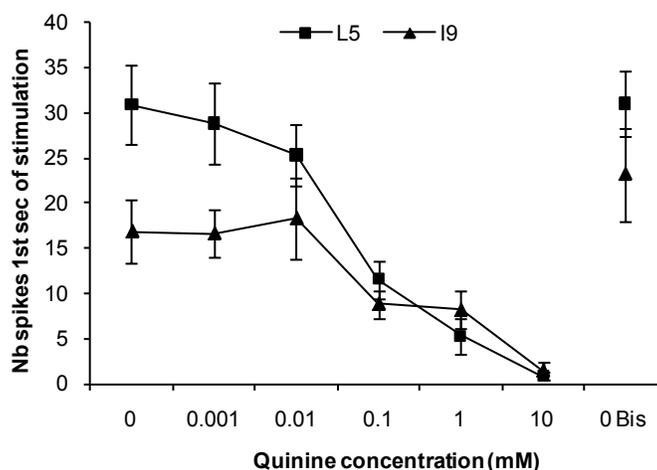


Figure 22. Electrophysiological dose-response curves obtained for fructose 35 mM mixed with different concentrations of quinine.

The recordings were made on the L5 (n = 10) and I9 (n = 9) sensilla. The different concentrations of quinine were tested in ascending order and another recording with fructose alone was done at the end of the series, to check for potential damages on the sensillum. This last stimulation is represented by the concentration called 0 Bis. Only sensilla responding to this last stimulation were included in the analysis. Error bars represent S.E.M.

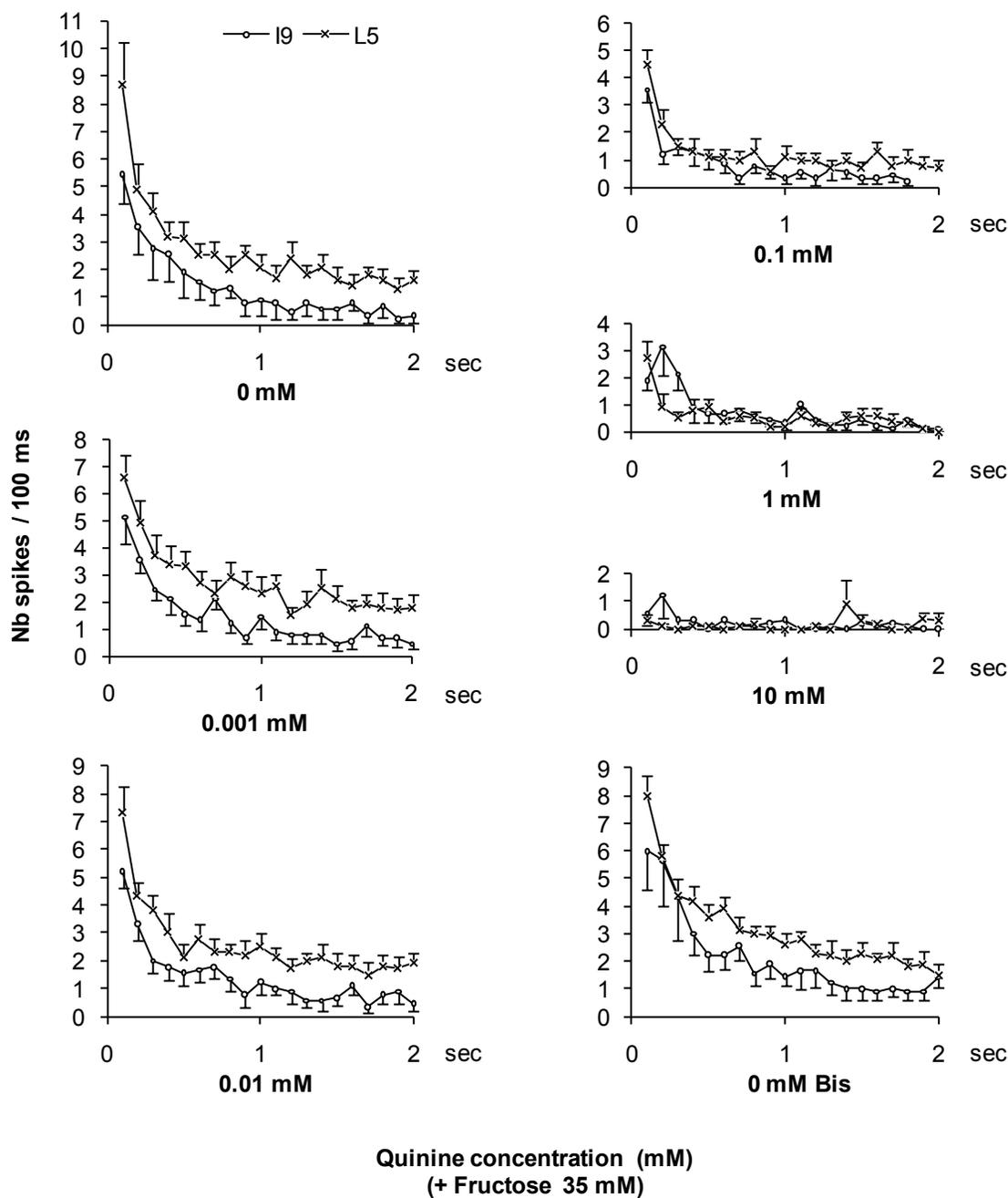


Figure 23. Number of spikes per 100 ms over 2 sec of stimulation with a mixture of fructose 35 mM and different concentrations of quinine.

These data indicate that quinine inhibits both the phasic part of the responses (first 200 ms) as well as the tonic responses (after 400 ms). Error bars represent S.E.M.

In order to estimate if the electrophysiological responses could be used to predict the behavioral activity, we plotted the electrophysiological responses across behavioral responses obtained with the same doses (Figure 24). As these data were not obtained on the same individuals, we compared the average consumptions obtained in the first set of behavioral data with the average electrophysiological responses recorded from L5 and I9 sensilla. These data were expressed as a percentage of the maximal response, *i.e.* the response for 35 mM fructose. These two set of data were highly linearly correlated ($R = 0.9681$). We note that the regression curve does not cross the Y axis at 0 but at about 12 % of the maximal response. This may represent a threshold under which the peripheral response does not induce any feeding response.

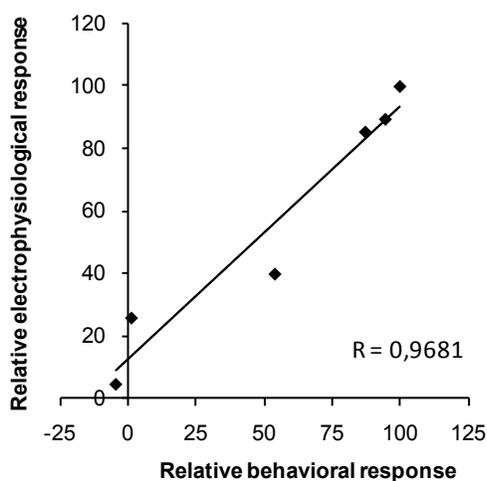


Figure 24. Linear correlation between the electrophysiological response and the behavioral response for quinine.

The responses are expressed in percentage of the maximal response (for fructose alone). The electrophysiological response is the number of spikes during the first second of stimulation, averaged on L5 and I9. The behavioral response is the averaged consumption in the MultiCAFE for all the data on quinine collected during the parameters adjustment.

The comparison of our behavioral results with our electrophysiological observations revealed a surprisingly good correlation with the inhibition on sugar detection rather than with the elicitation of a bitter-specific response. Most of the spikes recorded in this experiment were fired by S-cells (sugar-sensing cells). According to earlier work, the W cell is completely inhibited by 35 mM fructose and L1 cells do not respond to quinine or to fructose (the electrolyte, 1 mM KCl may elicit some spikes) (Meunier *et al.*, 2000; Hiroi *et al.*, 2002; Meunier *et al.*, 2003; Hiroi *et al.*, 2004). According to these authors and other work, bitter substances are

detected by L2 cells which express *Gr66a*. However, L-type sensilla are devoid of *Gr66a*-expressing cells, while I-type sensilla house one L2 cell that was expected to respond to the highest concentrations of quinine. Unexpectedly, it was not possible to detect the activation of the L2-cell in I-type sensilla or at least, it remained quite inactive since we obtained only a few spikes at these concentrations. In summary, the most conspicuous effect of quinine was to inhibit firing in the sugar cell. Such an inhibition is consistent with earlier observations on taste sensilla of the proboscis (Tanimura *et al.*, 1978; Rodrigues and Siddiqi, 1981) and of the leg (Meunier *et al.*, 2003).

4. Specificity of the inhibition

In order to determine if the inhibition of sugar detection by antifeedants was a general phenomenon or if some kind of specificity existed, we recorded the response of *w¹¹¹⁸* flies by stimulating L5 and S6 sensilla with sucrose 0.1 M alone and mixed with different antifeedant molecules (caffeine, lobeline, nicotine and strychnine) at 1 mM. Only the sensilla still responding to sucrose alone at the end of the experiments were kept for the analysis.

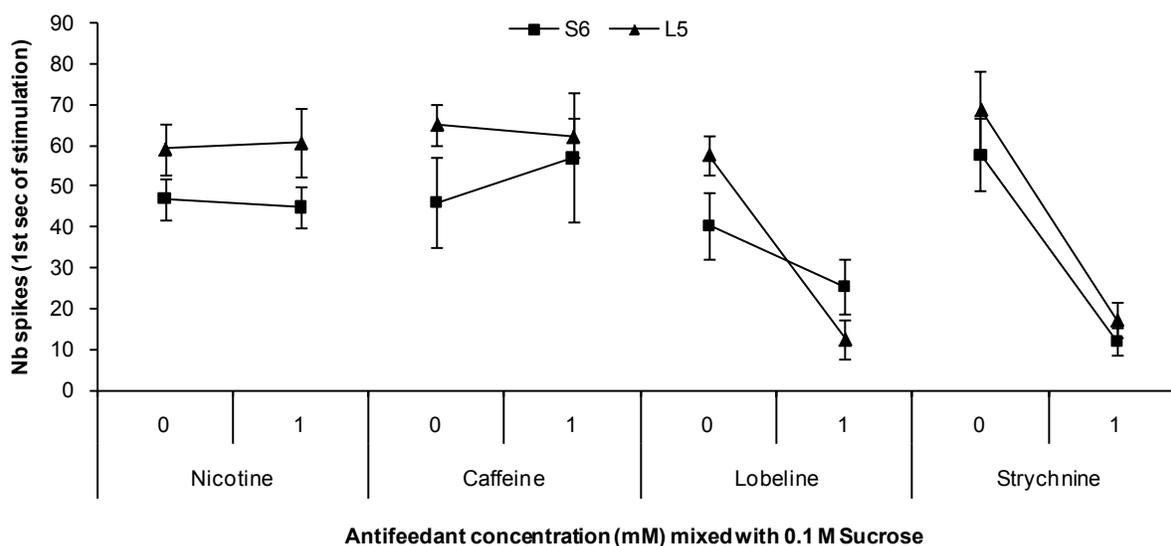


Figure 25. Specificity of sucrose inhibition by different alkaloids.

We stimulated L5 and S6 sensilla with sucrose 0.1 M alone or mixed with 1 mM nicotine, caffeine, lobeline or strychnine. Only the sensilla still responding to sucrose alone at the end of the experiments were included in the analysis. $n = 4-6$ for each substance. Error bars represent S.E.M.

The choice of the antifeedants was made on the basis of the deterrent potency ranking we obtained earlier with the MultiCAFE. We selected strychnine, lobeline, caffeine and nicotine, which were ranked in this order, strychnine being the most potent molecule. We could see that strychnine and lobeline elicited the greatest reduction of activity of the receptor cells (Figure 25), while caffeine and nicotine did not seem to have any effect at the tested concentration.

5. Test for a lateral interaction between the sugar and bitter cells

A. Electrophysiological inhibition of the S cell in L2-lacking flies

We exploited the UAS-Gal4 system to selectively kill the L2 cell in the taste sensilla, using a *Gr66a-Gal4* line to specifically target the bitter-sensing cells and a *UAS-DTI* strain as the lethal agent (Figure 26). Briefly, in the GR66a-expressing neurons of the F1 generation, the promoter of *Gr66a* is activated and allows the expression of the Gal4 protein. In turn, this protein activates the UAS promoter which elicits the expression of diphtheria toxin and thus the death of the cell. In the parental strains, either the Gal4 protein accumulates without effect (*Gr66a-Gal4*) or the UAS promoter is not activated because Gal4 is not present (*UAS-DTI*), which means that the L2 cell remains intact in both lines.

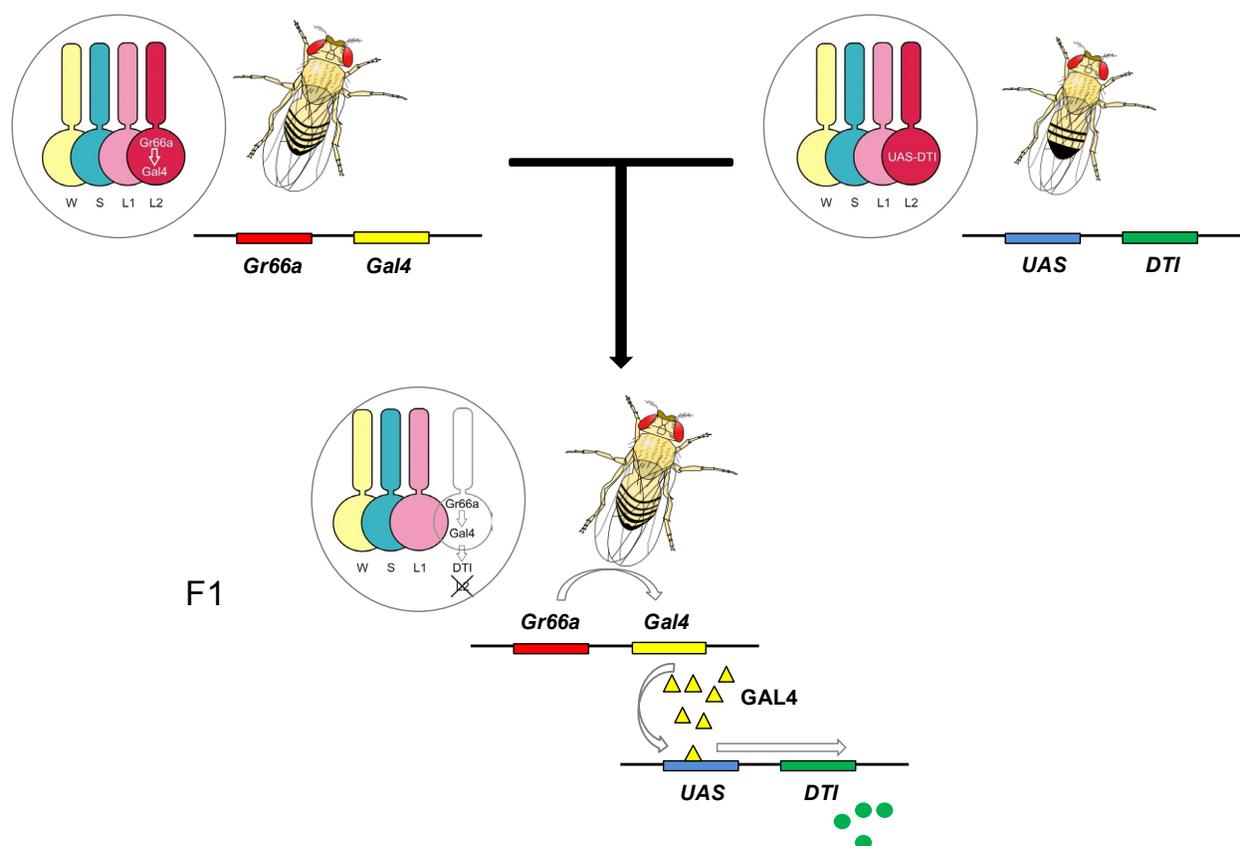


Figure 26. Selective elimination of the L2 cells using the UAS – Gal4 system.

Gr66a-Gal4 flies were crossed with a *UAS-DTI* (diphtheria toxin) strain, in order to target the bitter-sensing neurons (scheme modified from Ishimoto and Tanimura, 2004).

We built dose-response curves on L5 and S6 sensilla for sucrose (0.01, 0.1 and 1M), for strychnine (0.1, 1 and 10 mM) and for sucrose 0.1 M mixed with different concentrations of strychnine (0.1, 1 and 10 mM). The concentrations were presented in ascending order. For the mixtures, the sensilla were stimulated a second time, at the end, with the first solution tested. In this case, only the sensilla responding to the first and last stimulation were taken into account.

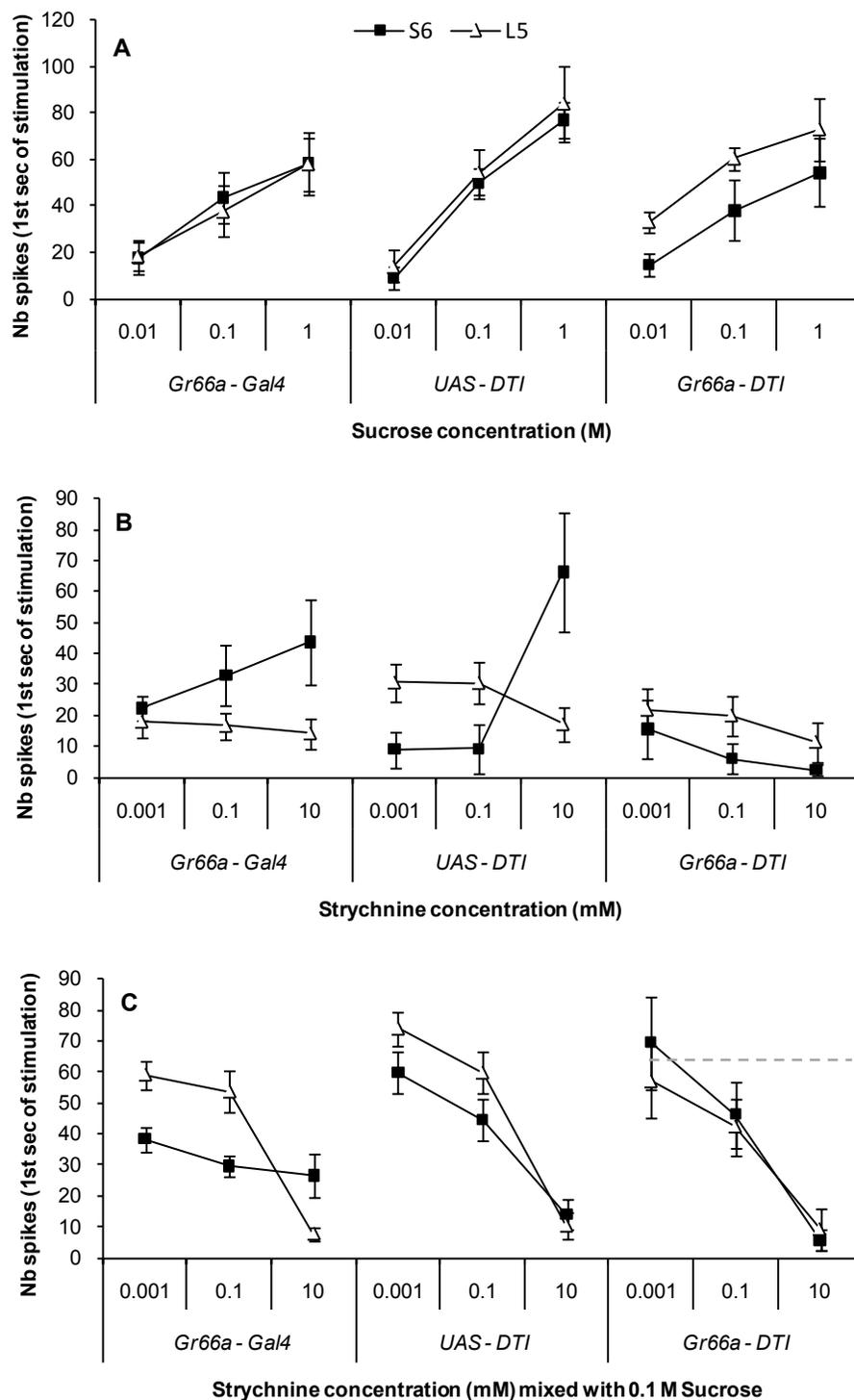


Figure 27. Electrophysiological responses of *Gr66a-Gal4*, *UAS-DTI* and *Gr66a-DTI* flies.

We stimulated S6 and L5 sensilla with A) sucrose, B) strychnine or C) a mixture of 0.1 M sucrose and different concentrations of strychnine. For the mixture recordings, the data corresponds to the total number of spikes, without sorting between the S and the L2 cells. The dotted line represents the expected response if sugar detection inhibition was due to an interaction between the S and the L2 cells. n = 5-7 for each curve. Error bars represent S.E.M.

First, the *Gr66a-Gal4* and *UAS-DTI* strains do not have any defect in sucrose detection (Figure 27A). For both strains and both sensilla tested (L5 and S6), the number of spikes elicited was positively correlated with the concentration of sucrose. Secondly, the profiles obtained on the S6 sensillum for strychnine showed that the parental strains did not seem to have trouble detecting strychnine either (Figure 27B). Moreover, our results on the L5 sensilla agreed with the previously postulated absence of response of the L-type sensilla to strychnine (Hiroi *et al.*, 2004). Finally, when L5 and S6 sensilla were stimulated with sucrose 100 mM mixed with different concentrations of strychnine, the activity of the receptor cells decreased when the concentration of strychnine increased (Figure 27C). These data confirm that the presence of strychnine inhibits the activity of the S cell, and this in both *Gr66a-Gal4* and *UAS-DTI*.

We crossed these two strains to obtain *Gr66a-DTI* flies. It's been shown previously that the S6 sensilla expressed *Gr66a*, which does not seem to be the case for L5 (Hiroi *et al.*, 2002). Thus, the constructions using *Gr66a-Gal4* should target the L2 cell in the S6 but not in the L5 sensillum. As expected, the L5 and S6 sensilla of *Gr66a-DTI* still responded to sucrose (Figure 27A). On the other hand, the low response of the S6 sensillum when stimulated with strychnine suggests that the crossing had the desired effect (Figure 27B). In this case as for the L5 sensilla, the remaining activity may come from the W cell.

The main goal of this work was to confirm or discard the possible involvement of the L2 cell in sugar inhibition. The L5 sensillum of the *Gr66a-DTI* flies still showed sugar inhibition when stimulated with the sucrose and strychnine mixture (Figure 27C). As we said earlier, the L2 cell of these sensilla should be intact, even in the *Gr66a-DTI* flies. However, we have shown that this sensillum did not respond to strychnine. In the S6 sensilla, which L2 cell expresses *Gr66a* and should thus be killed in the crossing, the activity of the sugar-sensitive cell was still inhibited when sucrose was mixed with strychnine (Figure 27C).

Even in the bitter-sensitive strains, we generally did not record many spikes from the L2 cell when we stimulated S6 sensilla with sucrose and high concentrations of strychnine. The Figure 28 shows typical recordings for sucrose, strychnine or the mixture of the two molecules on S6 sensilla of the *UAS – DTI* strain. The response to sucrose came from the S cell. When S6 sensilla were stimulated with strychnine alone, we observed a high activity of the L2 cell (small

spikes) and the W cell could fire large spikes in response to the electrolyte (KCl 1 mM). On the other hand, when sucrose and strychnine were mixed together, the activity of both the S (large spikes) and the L2 cell (small spikes) was much lower than when the sensilla were stimulated with the molecules separately. These observations are in favor of a reciprocal inhibition between sucrose and strychnine.

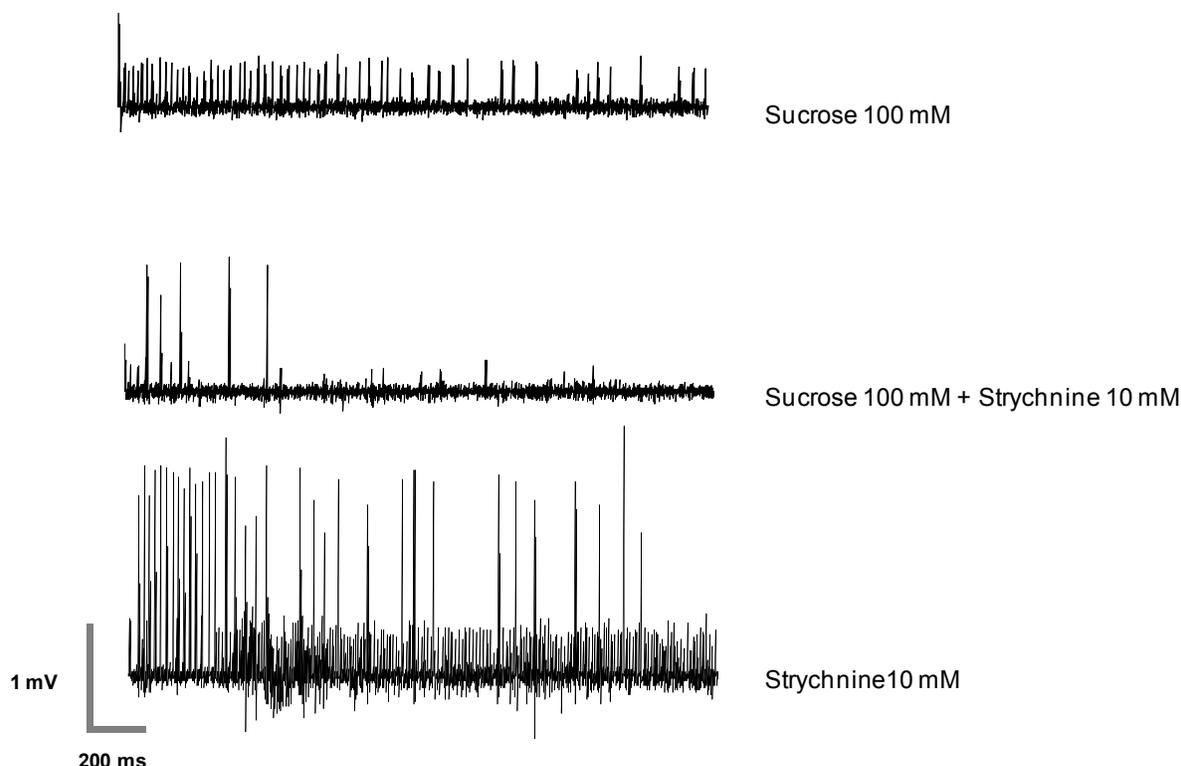


Figure 28. Electrophysiological recordings on S6 sensilla for sucrose, sucrose mixed with strychnine or strychnine alone, on the *UAS-DTI* strain.

For sucrose, the spikes come from the S cell. For sucrose mixed with strychnine, the large spikes should come from the S cell and the small spikes from the L2 cell. For strychnine alone, the large spikes might be fired by the W cell and the small spikes come from the L2 cell.

B. Inhibition of (sucrose + strychnine) consumption in L2-lacking flies

We then tested if the elimination of the bitter-sensing cell modified the feeding behavior of flies provided with sucrose and different concentrations of strychnine in the MultiCAFE. Briefly, emerged flies (~1-2 day old) were transferred to a freshly prepared food medium for 1 day and maintained in a rearing chamber at 25°C. The flies were first sexed (after numbing them

on ice) and the females were starved during 20 – 22 hours. Just before the experiment, the flies were introduced into the experimental boxes in groups of 20, without anesthesia. All the experiments were conducted at 25°C and ~70 % humidity. Each row of capillary tubes was filled with serial dilutions (0, 0.1, 1 and 10 mM) of strychnine mixed with 0.1 M sucrose and 0.125 mg / ml of blue food dye (n = 12). As a control series, we also tested a row of capillaries with only sucrose and the blue dye (n = 12).

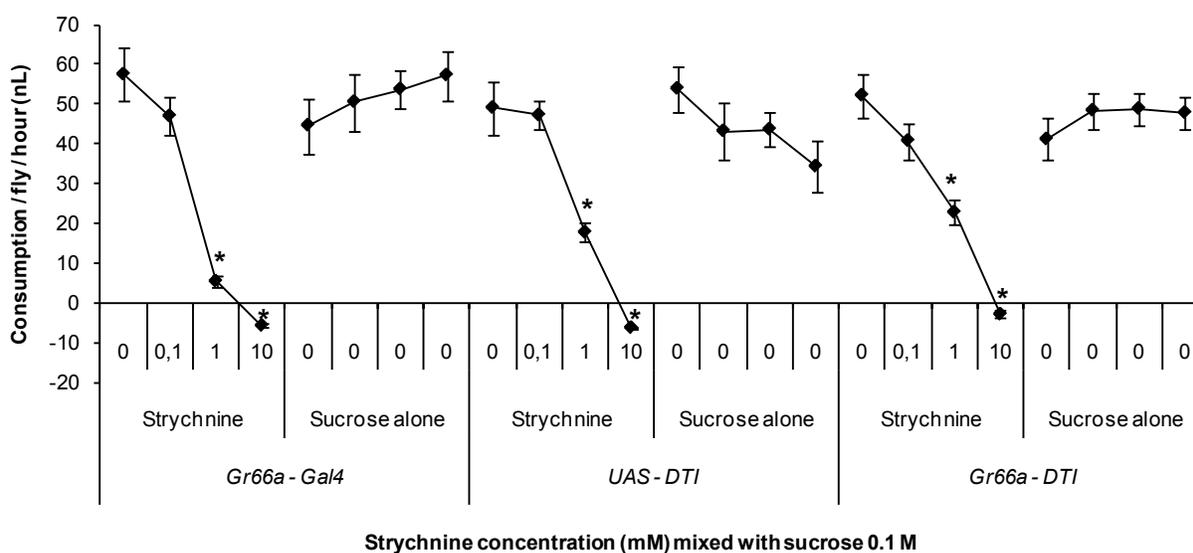


Figure 29. MultiCAFE dose-response profiles of the *Gr66a-Gal4*, *UAS-DTI* and *Gr66a-DTI* strains for sucrose alone or mixed with different concentrations of strychnine. n = 12 for each curve. Error bars represent S.E.M. On the curves, the asterisks represent concentrations for which the consumption is significantly different from the intake of fructose alone on the same curve (MANOVA, Profile analysis, $p < 0.05$).

We obtained similar curves for the parental lines, *Gr66a-Gal4* and *UAS-DTI*, and for the crossing strain *Gr66a-DTI* (Figure 29). When strychnine was added to sucrose, the higher was the concentration of strychnine, the lower was the consumption of the flies. For each strain, the dose-response profile obtained with strychnine was found to be significantly different from the sucrose control profile ($p < 0.001$, MANOVA). Moreover, we computed the total consumption by adding up the consumption in the 4 capillary tubes (Figure 30). We did not find any difference in the total consumption between the strains either for strychnine ($p = 0.7602$,

ANOVA) or for the sucrose control ($p = 0.2422$). Thus, these strains do not seem to differ in their hunger level.

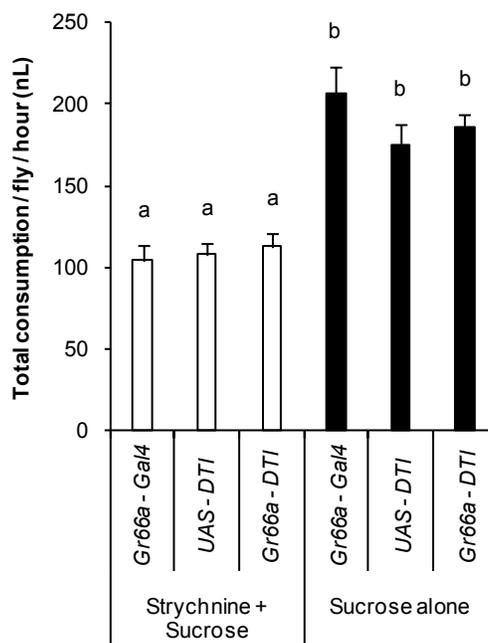


Figure 30. Total consumption for sucrose 0.1 M alone or mixed with different concentrations of strychnine. The total consumption was obtained by adding up the consumption in the 4 capillary tubes (see Figure 29). Error bars represent S.E.M. Data marked by different letters are significantly different (Bonferroni, $p < 0.01$).

6. Conclusion

We have shown that the behavioral data we obtained with the MultiCAFE for quinine was highly correlated with the response of the receptor cells. Quinine did not seem to activate much the bitter-sensing cell but had a strong inhibitory effect on fructose detection by the sugar-sensing cell. The correlation between these results and the consumption of the flies suggests that sugar detection inhibition could play a major role in the feeding behavior of the flies.

At a given concentration, inhibition of sugar detection by aversive molecules depends on the antifeedant tested. Indeed, among the four alkaloids we selected, two strongly inhibited the

response of the sensilla to sucrose (strychnine and lobeline) and the other two (caffeine and nicotine) did not seem to have any effect at the concentration tested. These results are in agreement with the bitter ranking we obtained with the MultiCAFE, in which strychnine and lobeline were the most potent molecules, while caffeine and nicotine were among the weakest ones. Masek and Scott (2010) showed that *D. melanogaster* do not discriminate among bitter compounds based on chemical identity in the PER assay. Instead, different deterrent compounds can be distinguished at a given concentration because some are more potent than others and thus less palatable. But the flies cannot make the difference between two antifeedants if the chosen concentrations give similar palatability. Caffeine and nicotine being less potent than the others according to our results in the MultiCAFE, we cannot exclude that these compounds may have an effect on sugar detection at higher concentrations.

Subsequently, we generated *Gr66a-DTI* flies, lacking the bitter-sensing cell, and we showed that the inhibition of sucrose detection by strychnine was not abolished by the absence of L2 cell. These results suggest that the L2 cell is not necessary for sugar detection inhibition. Thus, this phenomenon does not seem to be due to a lateral interaction between the sugar- and the bitter-sensing cells. Considering the fact that sugar detection inhibition by deterrent molecules also happens in L5 sensilla, an involvement of the L2 cell in this inhibition seemed unlikely. Indeed, L5 sensilla do not respond to aversive molecules (Meunier *et al.*, 2003). Thus, the L2 cell should not be activated in presence of deterrent compounds, which makes it improbable for this neuron to have an effect on the sugar-sensing cell.

When we correlated our MultiCAFE data with electrophysiological recordings, we did not record many spikes from the L2 cell after stimulation of I9 sensilla with fructose and high concentrations of quinine. We observed the same phenomenon when we stimulated S6 sensilla with sucrose and strychnine to test for a lateral interaction between the L2 and the S cell. Moreover, this phenomenon has been shown with different sugars or antifeedants on several insects (Shields and Mitchell, 1995a; Liscia and Solari, 2000; Jørgensen *et al.*, 2007). These findings suggest that a reciprocal inhibition of the L2 cell by sugars might exist. In the hamster, there seems to be a mutual inhibition between sucrose and quinine or denatonium in the nucleus of the solitary tract, but not between sucrose and caffeine (Li and Smith, 1996). Further observations are clearly needed to establish whether or not the presence of sugar in the stimulatory mixture reciprocally modulates the activity of the bitter-sensing cells.

Our MultiCAFE experiments on the *Gr66a* – *DTI* strain showed that the absence of bitter-sensing cell did not modify the feeding behavior of the flies towards sucrose mixed with strychnine. Even without detection of strychnine by the L2 cells, the inhibition of sucrose detection was still active and dose-dependent. In a previous study, Wang *et al.* (2004) used the PER assay to test *Gr66a-DTI* flies with sucrose 100 mM mixed with different concentrations of various antifeedants. They showed that the elimination of the L2 cell reduced but did not abolish the aversion towards these deterrent compounds. If we make the hypothesis that the remaining aversive response comes from sugar inhibition, their data suggest that this phenomenon is still present, at least partly, in absence of L2 cell, which supports our results. We cannot completely discard the possibility of a residual activity of the L2 cell in *Gr66a-DTI* flies. Nevertheless, this hypothesis seems unlikely considering our electrophysiological findings. Moreover, other strychnine receptors might exist on neurons distinct from the L2 cells in other sensilla of the fly's body, which could explain the remaining inhibition in the behavioral results. However, this hypothesis does not challenge our conclusions on the L2 cell, supported by single sensillum recordings.

Meunier *et al.* (2003) had suggested that both the activation of the L2 neuron and the inhibition of the S cell contributed to the repellency of bitter compounds and that sugar detection inhibition could play a major role in bitter taste coding. Taken together, our findings confirm these hypotheses. Moreover, we showed that these two mechanisms involved distinct receptor neurons and that sugar detection inhibition did not seem to be due to a lateral action of the bitter-sensing cell. In the introduction, we said that habituation could happen in parallel with a decrease in the peripheral sensitivity towards the molecule to which the insects have been exposed. If we could find a molecule to which fruit flies habituated and which had the two modes of action described here, then we could test if the decrease in sensitivity was due to both phenomena or to only one of them. Indeed, a reduction in the inhibition of sugar detection following a prolonged exposure to the antifeedant would also contribute to increase the palatability of the food and, thus, the consumption.

IV. Experience-induced modulation of feeding

1. Introduction

The aim of this PhD was to test the hypothesis of a negative correlation between the sensitivity to deterrent molecules and the efficiency of the detoxification systems, with a direct influence on the taste organs' sensitivity (Figure 31). This hypothesis was largely based on the observations made by John Glendinning. Glendinning has devoted a large part of his research to a better understanding of the changes in the feeding behavior of insects caused by a prolonged exposure to secondary compounds. Exposure to a secondary compound can lead to a rapid rejection response due to the activation of bitter-sensitive gustatory neurons (Glendinning and Hills, 1997) or to a rapid post-ingestive mechanism (Glendinning, 1996). In case of prolonged or repeated exposure, slower post-ingestive processes may occur. In particular detoxification enzymes can be induced (among which the P450s), which in turn leads to an increase in the consumption of the deterrent molecule (Glendinning and Slansky, 1995; Snyder and Glendinning, 1996).

The increased consumption of an antifeedant following prolonged exposure may also be due to a decrease in the taste sensitivity to this molecule. This phenomenon can be mediated peripherally, through the desensitization of the deterrent cell (Glendinning *et al.*, 1999). After the withdrawal of the antifeedant from the diet, the bitter-sensing cell slowly recovers and goes back to a higher activity (Glendinning *et al.*, 2001b; Glendinning *et al.*, 2001a). The decrease in sensitivity to a compound after prolonged exposure can also be mediated centrally, through a modulation of the behavior triggered by the central nervous system when integrating the peripheral response to this molecule (Glendinning *et al.*, 2001a; Glendinning *et al.*, 2006). In our work, we wanted to see if sensitivity and detoxification were linked and if the induction of P450 enzymes could directly induce a decrease in sensitivity.

Some cells might detect various deterrent compounds through different signaling pathways (Glendinning and Hills, 1997; Glendinning *et al.*, 2002). In this case, the exposure to the antifeedant may desensitize specifically the corresponding signaling pathway, without modifying the detection of the molecules processed through other pathways (Glendinning *et al.*, 1999; Glendinning *et al.*, 2001b). The central nervous system could distinguish between various

antifeedants through different populations of bitter-sensing cells in the sensilla, responding to different molecules. However, another hypothesis concerning the discrimination of deterrent compounds emerge from these observations: antifeedants processed through different signaling pathways might elicit responses with different discharge patterns and the central nervous system could use this temporal coding to distinguish between them (Glendinning *et al.*, 2002; Glendinning *et al.*, 2006).

Habituation can generalize to higher concentrations of the exposure molecule and to other substances (Glendinning and Gonzalez, 1995). However, this generalization is specific and does not occur for all compounds. For example, the specific desensitization of one signaling pathway can cause a specific generalization, with an increase in the consumption of the molecules processed through the desensitized pathway but no change in the intake of molecules processed along other signaling pathways (Glendinning *et al.*, 2002). Moreover, generalization can be asymmetrical, with habituation to one compound leading to habituation to another one but not the reverse situation (Glendinning and Gonzalez, 1995). This generalization phenomenon may be explained by postulating that the deterrent molecules are processed along different sensory signaling pathways but also by the hypothesis that they induce partially overlapping sets of detoxification enzymes.

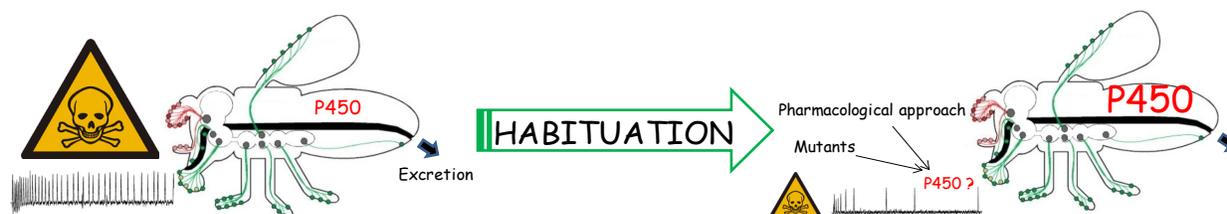


Figure 31. Schematic representation of the initial PhD project.

In this work, we tested if exposure to caffeine would induce habituation in adult flies. Then, we had planned to study the expression of detoxification enzymes, with and without habituation, using RNA microarrays, in order to look for detoxification enzymes which

expression could be modified by habituation. The final step would have been to try to correlate those two parts by pharmacologically activating or inhibiting (or under- or over-expressing) the candidate detoxification enzymes found in the second step and see if peripheral sensitivity was modified. This last part was more speculative because it depended on the success of the two previous ones.

Here we describe our attempts to set up a situation of habituation. If we had succeeded in establishing habituation, we could have studied the changes in the expression of the detoxification enzymes and also the involvement of the antifeedants' two modes of action in this phenomenon. Indeed, as we said before, habituation generally goes with a decrease in taste sensitivity and it would have been interesting to know if both effects of the antifeedants were affected or not.

2. Attempt to set up a paradigm of habituation with caffeine

The aim of this series of experiments was 1) to expose adult flies to a medium supplemented with caffeine for a certain amount of time and 2) to test the flies in the MultiCAFE to look for potential changes in their consumption of this molecule. We decided to expose adult flies to caffeine and not to raise the flies on a caffeine-containing medium to avoid the confusion between developmental effects and habituation. We chose caffeine because the majority of the P450 enzymes involved in caffeine detoxification have already been described (Willoughby *et al.*, 2006), which represented a real advantage for the second part of the study.

For caffeine exposure, we tested different durations (24 and 48 h) and different concentrations (0.01, 0.1, 1 and 10 mM). The flies were raised on normal medium (Figure 32). Newly emerged flies were sexed and then transferred by groups of 20 on the caffeine-containing medium (agar, sucrose 5 %, yeast extract 5 %, blue dye and caffeine). Control flies were transferred on a similar but caffeine-free medium. The presence of blue dye in the abdomen of the flies indicated that they had consumed the medium. After exposure to caffeine, the flies were starved for 20-22 h in plastic vials provided with humidified filter paper and tested in the

MultiCAFE. The 6 capillary tubes contained fructose 35 mM and different concentrations of caffeine (0, 0.001, 0.01, 0.1, 1 and 10 mM).

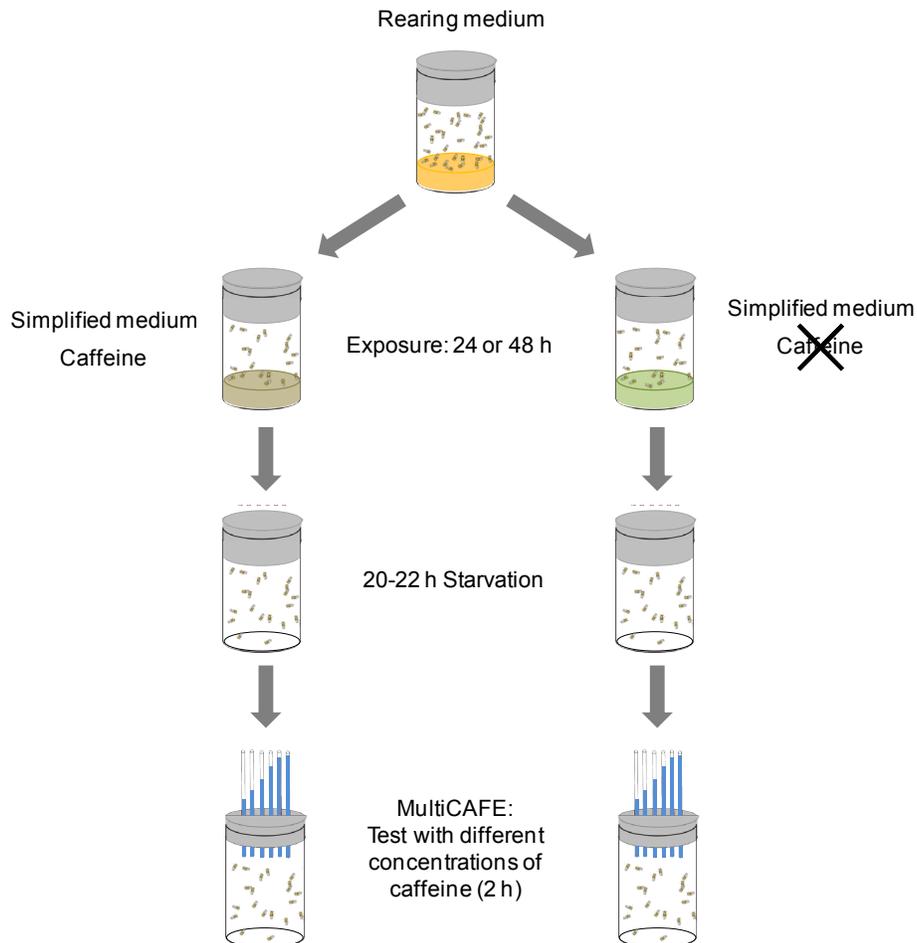


Figure 32. Habituation protocol.

Newly emerged flies were transferred on a simplified medium with or without caffeine. They were then starved and tested in the MultiCAFE with different concentrations of caffeine.

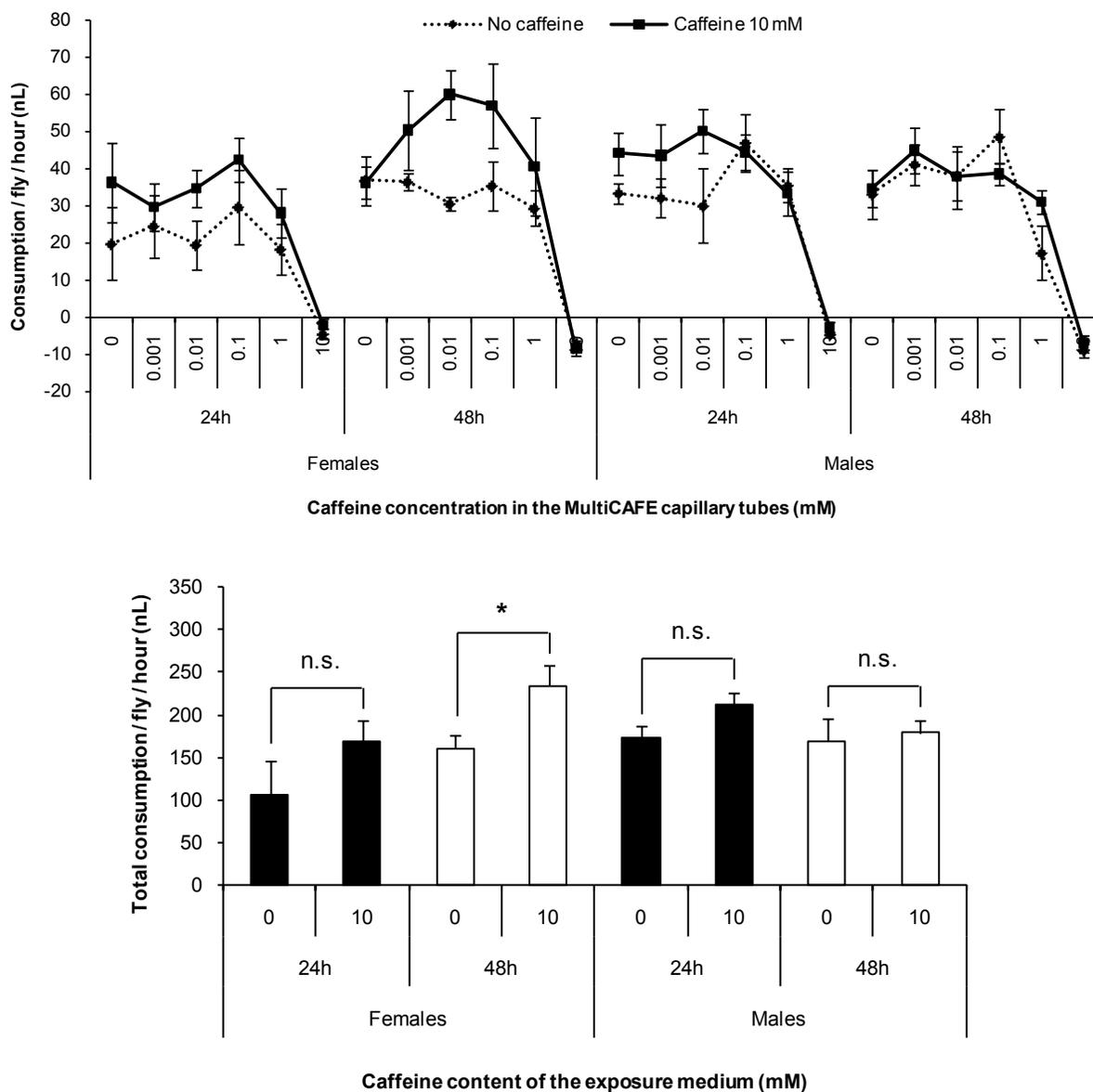


Figure 33. Caffeine dose-response curve and total consumption of males and females, after 24 or 48 h of exposure to a caffeine-free medium or a medium containing 10 mM caffeine.

The total consumption was obtained by adding up the consumption in the 6 capillary tubes provided to the flies. $n = 5$ for each curve. Error bars represent S.E.M. Differences between total consumptions were calculated using ANOVAs (*: $p < 0.05$).

Only the results for an exposure to 10 mM caffeine are presented here as this concentration was the only one to show some effect (Figure 33, $n = 5$ for each curve). After 48 h of exposure to caffeine 10 mM, females seemed to feed more on mid-range concentrations of this molecule than flies which have never been exposed to it (MANOVA, $p = 0.0456$). Nevertheless, these results do not represent a clear situation of habituation. Different hypotheses can be considered to explain this absence of clear results. First, the concentrations tested might be too low to cause habituation. However, if the concentration is too high, the flies may not eat much or even at all. Thus, habituation should occur more at mid-range concentrations than at highly deterrent concentrations. Weaker stimuli are generally better to elicit habituation than strong ones (Thompson and Spencer, 1966; Szentesi and Bernays, 1984). A second hypothesis could come from the starvation undergone by the flies before the test. Starvation is necessary to increase the feeding motivation of the flies but this treatment might have unknown effects on the habituation phenomenon, especially when it occurs right after the exposure to the antifeedant. As a conclusion, we can say that the preliminary experiments to set up a habituation paradigm showed us the unwieldiness of the protocols but did not give satisfactory results.

3. Modulation of the P450 activity with metyrapone

The main hypothesis of the PhD thesis was a correlation between a decrease in taste sensitivity to an antifeedant and an increase in the activity of the corresponding P450 detoxification enzymes after an exposure to this antifeedant. Metyrapone is a pharmacological inhibitor of the P450 enzymes. We applied this pharmacological agent to the taste sensilla in order to test the potential involvement of P450s in the electrophysiological response (or the response shutdown) to antifeedants.

We did a first stimulation with fructose 35 mM on L5 sensilla. Then the sensillum was bathed during 2 min with a solution of quinine 50 mM ($n = 6$) or quinine 50 mM + metyrapone 50 mM ($n = 5$) or metyrapone 50 mM alone ($n = 2$) or Ringer (87 mM NaCl, 5.4 mM KCl, 3 mM $\text{CaCl}_2\text{H}_2\text{O}$) ($n = 2$). Finally, we stimulated again the sensillum with fructose 35 mM at different time intervals (1, 3, 5, 7, 10, 15, 20 and 30 min) to observe the recovery of the response. The concentration of quinine and the exposure duration were determined by preliminary results obtained by Ligia Borges, a Brazilian researcher who came to our laboratory for a sabbatical in

order to learn how to do single-sensillum recording. The concentration of metyrapone was chosen according to Maïbèche-Coisne *et al.* (2004), who had already used metyrapone as an inhibitor of degrading enzymes in the sensilla of scarab beetles. They tested metyrapone at 10 and 100 mM and we chose 50 mM as an intermediate concentration.

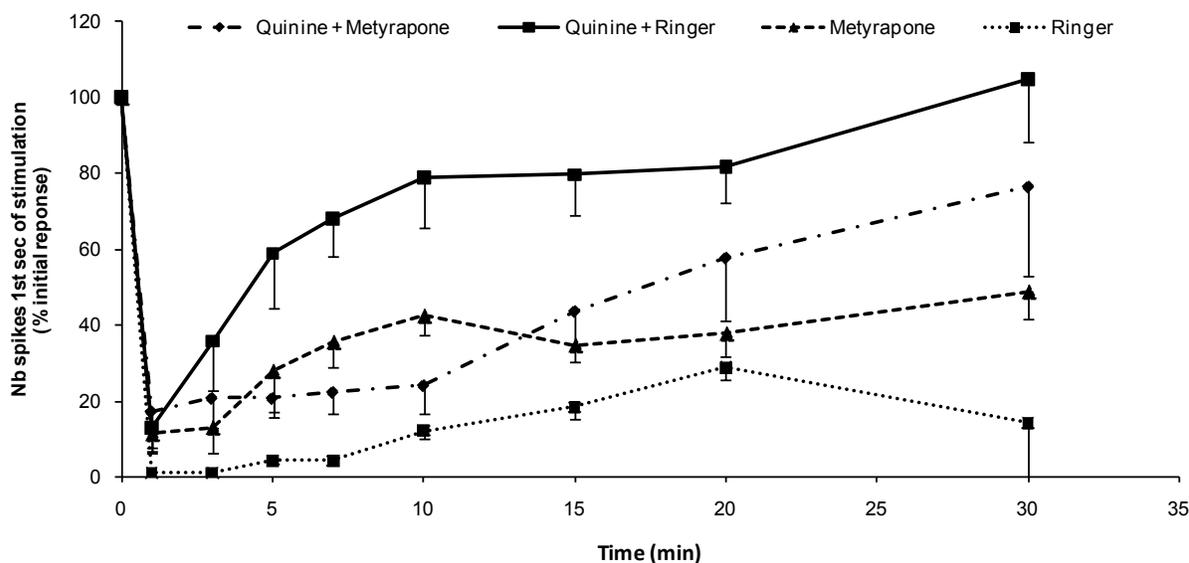


Figure 34. Recovery experiment.

We stimulated L5 sensilla with fructose 35 mM then we bathed them with quinine 50 mM ($n = 6$) or quinine 50 mM + metyrapone 50 mM ($n = 5$) or metyrapone 50 mM alone ($n = 2$) or Ringer ($n = 2$) during 2 min. Finally, we observed the recovery of the sensilla by stimulating them with fructose 35 mM at different time intervals. Error bars represent s.e.m.

After an exposure to quinine, we observed an inhibition of the response to fructose which led to a very slow recovery of the response to this sugar (about 30 min) (Figure 34). Concerning the mixture quinine + metyrapone, the hypothesis was that metyrapone would inhibit the activity of degrading enzymes, thus the quantity of quinine in the sensillum would remain high for a longer time and the recovery of the response to fructose would be slower. It is indeed what we observed. This result seemed to suggest that the P450 were involved in the degradation of antifeedants in the sensilla. As controls, we bathed the sensilla with metyrapone alone or Ringer solution. Although we do not have many repetitions for both treatments, the results we obtained are quite confusing. These solutions were not supposed to have any effect on the response to fructose but the electrophysiological recordings showed an inhibition of the response to this

sugar even greater than for quinine. Bathing the sensilla in Ringer solution for 2 min may damage the receptor cells by disturbing their osmotic pressure.

Before going further, we decided to discard a potential effect of metyrapone on sugar detection. We built an electrophysiological dose-response curve for sucrose 50 mM mixed with either quinine or metyrapone. As expected, the response of the L5 sensilla to sucrose decreased with increasing concentrations of quinine (Figure 35). What was not expected was to observe a lower but similar effect of metyrapone on sucrose detection. As P450 enzymes are not supposed to be involved in the response to sugars, these results are difficult to understand.

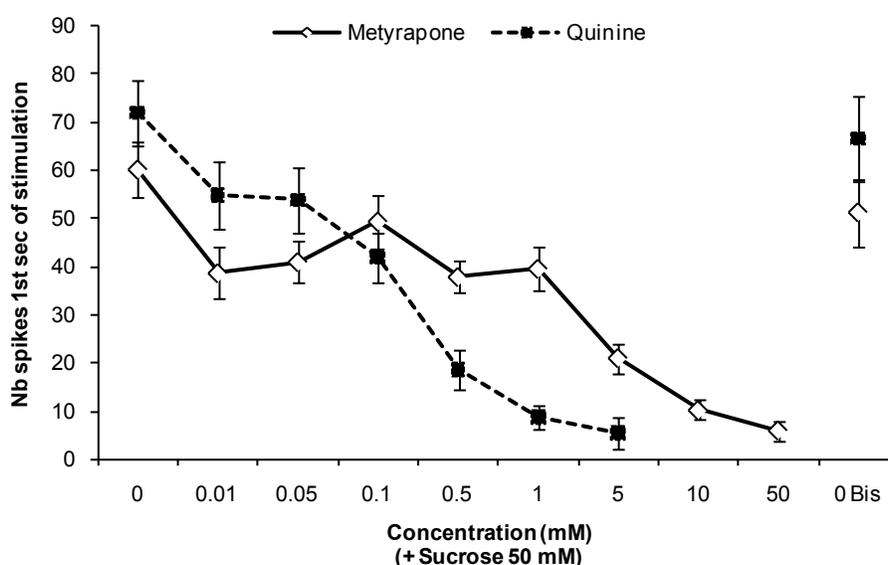


Figure 35. Inhibition of sugar detection by quinine and by metyrapone.

L5 sensilla were stimulated with sucrose 50 mM mixed with quinine or metyrapone at different concentrations. 0 Bis is a stimulation with sucrose alone at the end of the experiments to check for potential damage on the cell. Only the sensilla responding to this stimulation were taken into account. $n = 10$ for each curve. Error bars represent S.E.M.

4. Conclusion on the habituation experiments

The initial PhD project was based on the hypothesis that a negative correlation existed between taste sensitivity and activity of detoxification enzymes. The first step of this project was to determine a protocol to establish a situation of habituation causing changes in the peripheral taste sensitivity towards the chosen molecule. We chose caffeine because of the available data on

the enzymes involved in caffeine detoxification and we tested different exposure durations and concentrations. However, we could not find a clear habituation to caffeine in our results. Moreover, we used a pharmacological agent, metyrapone, in order to determine if detoxification enzymes were present in the taste sensilla and involved in the detection of deterrent molecules. During this study, we discovered that metyrapone had an inhibitory effect on sugar detection and thus, would be difficult to use in our experiments.

The absence of clear habituation results, the unwieldiness of the protocols, the problems concerning the effect of metyrapone on sugars and the lack of time to establish a reference situation led us to modify the PhD project. That is why we decided to further study the peripheral interactions between sugars and antifeedants. Indeed, we already had stable preliminary results to study this intriguing phenomenon.

5. Adaptation to sugars

Experience-induced modifications of feeding behavior have generally been studied for antifeedants but we can wonder if this phenomenon also occurs for sugars. Here we cannot use the term habituation because the consumption of sugar is vital and the absence of sugar in the food decreases or even abolishes feeding. Thus, insects are constantly exposed to sugar in their food. However, the type of sugar present in the food may have an effect on the perception or the preference towards the sugars consumed later on. These potential experience-induced modifications of feeding preferences have never been studied in *D. melanogaster*.

The following experiments were done in collaboration with Linda M. Kennedy and Kristina M. Gonzalez (Neuroscience Laboratory, Lasry Center for Bioscience, Clark University, Worcester, MA 01610-1477 USA). Linda Kennedy did a 1 month sabbatical in our laboratory. Kristina Gonzalez, her PhD student, came to our laboratory during 2 months to learn how to get electrophysiological recordings on *D. melanogaster* and how to use the MultiCAFE assay. She did most of the following experiments during her stay. The statistics used for this series of experiments were done by Kristina Gonzalez in Linda Kennedy's laboratory. This project was financed by Clark University.

The aim of the experiments was to see if experience-induced changes in sugar taste sensitivity occurred in *D. melanogaster*, and if so, where these changes took place. In order to answer those questions, flies were raised on a medium containing either fructose or glucose. Then, we measured their feeding behavior towards fructose or glucose and the electrophysiological response of sugar cells to those molecules.

A. Previous results obtained in Linda Kennedy's laboratory

Before coming to our laboratory, Linda Kennedy's team had done this experiment on Oregon R flies, using the two-choice test with the blue and red dyes. The 96 microwells of the plates contained either 1% agar alone or mixed with a sugar at a given concentration, in alternating order. The flies, reared either on 555 mM glucose or the approximately iso-stimulatory concentration of 139 mM fructose, were placed in the boxes for 2 h, in the dark. They were then killed with liquid nitrogen and the flies with a blue, red or purple abdomen were counted, in order to calculate the index, which was then transformed in percentage.

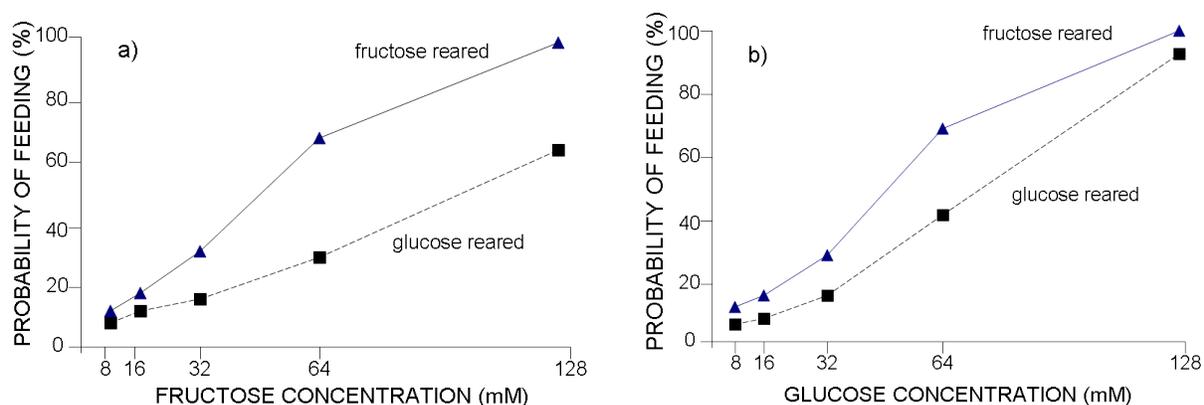


Figure 36. Previous results obtained in Linda Kennedy's laboratory.

Flies were raised on fructose 139 mM or glucose 555 mM and then tested with different concentrations of fructose or glucose in the colored wells test.

They found that the rearing medium had an effect on the choice of the flies in their assay (Figure 36). At low concentrations of fructose and glucose (8 and 16 mM), there was no

preference for these sugars compared to plain agar. This suggests that these concentrations may be too low for the flies to discriminate them. However, for higher concentrations and for both fructose and glucose, the fructose reared flies seemed to have a higher probability to choose the sugar over plain agar than glucose reared flies ($p = 0.0001$, Regression Analysis). Especially for the mid-range concentrations tested, differences in consumption between fructose and glucose reared flies were clear.

B. Changes in fructose or glucose consumption following exposure to these sugars

Canton S flies were raised on a medium containing either 139 mM fructose or 740 mM glucose (control). To check for any variation in consumption due to differences in the caloric value of the media, another group was raised on 740 mM fructose as a second control. This control relies on the assumption that equimolar solutions of fructose and glucose are supposed to be isocaloric (Southgate and Durnin, 1970).

Emerged flies were placed upon fresh medium (the same as the one they were raised on) for 24 h before being starved 20-22 h. Flies were then tested in the MultiCAFE assay (as described before) in groups of 40 (20 males and 20 females) with at least 10 repetitions per treatment. The capillary tubes were filled with either glucose or fructose (0, 8, 16, 32, 64 and 128 mM) mixed with the blue dye.

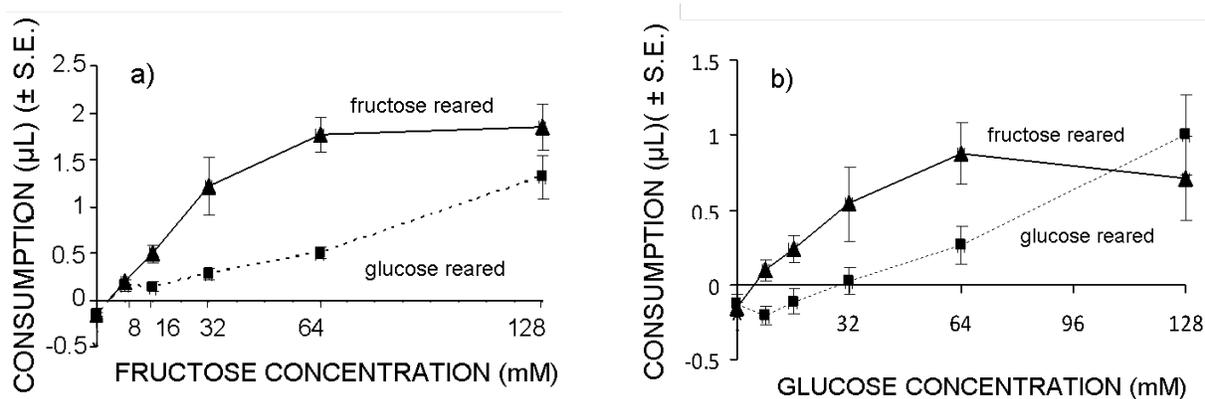


Figure 37. Influence of the sugar used in the rearing medium on the preference towards fructose or glucose. Flies were raised on fructose 139 mM or glucose 740 mM and then tested with different concentrations of fructose or glucose in the MultiCAFE. $n =$ at least 10 for each curve. Error bars represent S.E.M.

Here again, the rearing medium affected the consumption of sugars. At the higher and lower concentrations there were no differences in consumption, while there were clear differences between fructose- and glucose-reared flies at midrange concentrations (Figure 37). Flies reared on 139 mM fructose consumed significantly more fructose ($p = 0.0001$, Repeated Measures ANOVA) and glucose ($p = 0.003$) than the flies reared on 740 mM glucose. Flies reared on equimolar 740 mM fructose did not consume any measurable volume of fructose at any of the test concentrations.

C. Modulation of the electrophysiological response for fructose and glucose

In order to see if the taste receptor cells were involved in experience-induced changes in taste preferences, we recorded the peripheral electrophysiological response to sugar of the flies raised on the different media aforementioned. We chose to record the response of L-type sensilla (L3, L5 or L7) and this for two reasons. First, their sugar cell seems to give better responses to sugars than the sugar cell present in S- or I-type sensilla (Hiroi *et al.*, 2002). Secondly, they are the most readily accessible sensilla on the proboscis.

We built dose-response curves for fructose and glucose using 4-5 days old flies ($n = 10$ for each treatment). 10 mM choline chloride was used as an electrolyte. This concentration of choline chloride elicits water cell action potentials, but not sugar cell action potentials (Tanimura and Shimada, 1981). The stimulating electrode was brought into contact with a sensillum tip for a 5 s stimulus presentation. The sensillum was stimulated with each concentration of fructose or glucose 3 times, and the averages of the responses were used for data. We waited 2 min between stimuli to avoid sensory adaptation. Prior to each stimulation, fresh fluid was drawn to the pipette tip with a piece of filter paper to ensure that the same concentration of testing solution was presented each time.

We then plotted the number of spikes during the first 100 ms according to the 5 concentrations tested (8, 16, 32, 64 and 128 mM). The action potentials of individual cells were differentiated using our custom designed computer software program, dbWave (Marion-Poll and van der Pers, 1996), according to their relative amplitudes. The largest amplitude action

potentials, measured peak to trough, were identified as those of the sugar cell, as previously determined by Fujishiro *et al.* (1984).

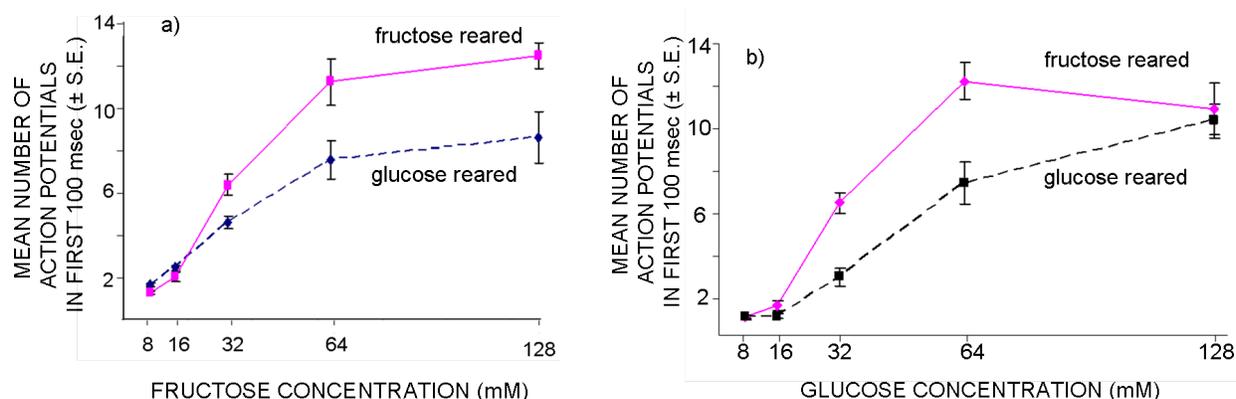


Figure 38. Change in the taste sensitivity towards glucose or fructose according to the sugar present in the rearing medium.

Flies were raised on fructose 139 mM or glucose 740 mM and then the activity of their L5 sensilla in response to different concentrations of fructose or glucose was recorded. $n = 10$ for each curve. Error bars represent S.E.M.

As well as in the behavioral assays, we found an effect of the rearing medium on the response of the L-type sensilla to sugars. There was a significant interaction between the rearing medium and the sugar receptor cell firing rate in response to both fructose (Figure 38, $p = 0.026$, Repeated Measures ANOVA), and glucose ($p = 0.003$). The sugar-sensing neuron of fructose-reared flies fired significantly more spikes in the first 100 ms than the S cell of glucose-reared flies, and this in response to both glucose ($p = 0.006$, Repeated Measures ANOVA) and fructose ($p = 0.002$).

We can see that the MultiCAFE behavioral curves and the electrophysiological data look very alike. The volumes consumed in the MultiCAFE and the number of spikes recorded in the first 100 ms were significantly and positively correlated for fructose tested flies ($r = 0.69$, $p = 0.0001$, Multivariate fit) and glucose tested flies ($r = 0.49$, $p = 0.0001$).

D. Discussion on sugar experience-induced modifications

The results we obtained show that flies reared on 139 mM fructose consumed more fructose and glucose, especially at the mid-range concentrations tested, than those reared on 555 mM or 740 mM glucose. The same results were found for two strains of *D. melanogaster*, Oregon R and Canton S, and in two behavioral paradigms, the colored wells test (where sugars are mixed with agar) and the MultiCAFE (where sugars are in solution).

The sugar routinely used in the rearing media for *D. melanogaster* is glucose. It is possible that the flies exposed to 139 mM fructose consumed more in the MultiCAFE assay because they were hungry after being reared on a medium that, while approximately iso-stimulatory, had a lower calorie density than the 740 mM glucose one. The sugar in the medium was thus changed to an equimolar concentration of 740 mM fructose. If the difference of consumption between the flies raised on 139 mM fructose and on 740 mM glucose was due to hunger, flies raised on 740 mM fructose should feed in about the same proportions as flies raised on 740 mM glucose. However, *D. melanogaster* did not eat any measurable amounts of sugar at any concentration after being exposed to 740 mM fructose. Therefore it is difficult to conclude about the hunger state of the flies raised on 139 mM fructose.

Evans (1961) reared blowflies, *Phormia regina*, on a medium of yeast, milk, agar and water, or the same medium supplemented with 100 mM glucose or 100 mM fructose. Blowflies reared on the glucose medium showed significantly greater sensitivities in the PER assay, when their tarsal taste sensilla were stimulated with glucose or fructose. Those reared on the fructose medium showed significantly lower sensitivities than blowflies raised on the plain medium. However, Cohen and Sturckow (1971) obtained different results. They raised flies on a rearing medium or on the same medium with 250 mM glucose or fructose. In their study, the blowflies reared on the glucose-containing medium showed significantly lower sensitivities in PER responses when tarsi taste sensilla were presented with glucose and sucrose, while the sensitivity to fructose remained unchanged. Those reared on the fructose-containing medium showed small decreases in sensitivity that were not statistically significant. Differences among results may be due to many factors among which differences in media, species, or concentrations of sugars.

The electrophysiological response of the sugar-sensing receptor neurons tested was similar in shape to the dose-response curves obtained for both the colored wells and the

MultiCAFE behavioral tests. The firing rate of the sugar taste receptor neurons in response to mid-range concentrations of fructose or glucose was significantly greater for fructose reared flies than for glucose reared flies. In addition, the MultiCAFE behavioral data and the electrophysiological data from the receptor cell obtained for Canton S flies were positively correlated. This suggests that the experience-induced modifications in the feeding behavior of the flies resulted, at least partly, from changes in the peripheral taste system sensitivity.

However, we cannot rule out experience-induced changes in the central nervous system as well. Corresponding peripheral and central nervous system changes have been shown in humans: subjects repeatedly exposed to novel taste stimuli showed concurrent increases in functional magnetic resonance imaging pixel activations and psychophysical estimations of stimulus intensities over several weeks of exposure to the stimuli (Faurion *et al.*, 1998). Moreover, sodium restriction during adulthood in rats transiently changes the electrophysiological response of the taste receptor cells (Wall and McCluskey, 2008) while sodium restriction during embryonic development of rats leads to modifications in the morphology of afferent neurons in the primary solitary nucleus of the brainstem (Mangold and Hill, 2007; May *et al.*, 2008).

In conclusion, *D. melanogaster* flies reared on fructose and glucose seem to undergo changes in their taste system leading to modifications of their perception of sugars. The results we obtained suggest that these changes are mostly peripheral and one of the hypotheses could be a differential modulation of the taste receptors expression or sensitivity. However, some changes in central mechanisms remain possible.

Our experiments on caffeine did not give very conclusive results. However, this initial project gave us the opportunity to collaborate in this study on experience-induced feeding behavior modifications related to sugars. This work allowed us to further validate our MultiCAFE behavioral test by comparing results obtained with this assay to data coming from the colored wells test and electrophysiological recordings. Moreover, we showed that the sugar used in the rearing medium had an effect on the adult flies' perception of sugars.

V. General conclusion on the PhD project and perspectives of the study

1. Conclusion

The initial PhD project was based on the hypothesis that, in the habituation process, a negative correlation existed between peripheral sensitivity and activity of the detoxification enzymes. A quantitative multiple-choice assay was designed to show modulations of feeding behavior consecutive to habituation. Indeed, the most commonly used feeding preference assays in *D. melanogaster* give qualitative more than quantitative results. Moreover, their design does not allow more than two-choice experiments, which represents a limitation for screening molecules. During this PhD thesis, we developed the MultiCAFE, a multiple-choice consumption test modified from Ja *et al.* (2007). In this setup, flies are provided with different capillary tubes filled with various solutions and their consumption is measured after two hours. With this test, we have shown first that fly density had an influence not on the feeding choices of the flies but on the volume they consumed. Indeed, the shape of the curve was similar for densities of 10, 20 40 or 60 flies but the individual consumption was higher for lower densities. Secondly, we have found that the order of presentation of the different solutions could have a slight effect on the consumption but did not seem to impair discrimination. Thirdly, according to the spacing of the capillary tubes, access from one tube to another could seem to facilitate feeding and competition seemed more or less likely. However, the spacing of the capillaries did not have any effect on the dose-response profiles we obtained for quinine. Moreover, we observed a similar sensitivity when we used this assay in no-choice, two-choice and multiple-choice experiments. Thus, the flies do not seem to have more trouble discriminating the solutions in multiple-choice tests than in simpler ones. We then used this assay to test 8 alkaloids and rank these molecules according to their bitter potency: strychnine > lobeline > berberine > theophylline > quinine > caffeine > papaverine > nicotine. To finish, we tested mutants previously reported to have caffeine-detection defects and we showed that, in addition to caffeine-detection impairments, this strain had also a lower general consumption compared to the control strain.

We found a good correlation between our MultiCAFE behavioral data for quinine and the electrophysiological response of labellar sensilla. Quinine had a very strong dose-dependent

inhibitory effect on fructose detection. Indeed, antifeedants can have two non-exclusive modes of action on the taste receptor cells: they can activate the bitter-sensing cells or inhibit sugar detection by the sugar-sensing cells. Intriguingly, this inhibition phenomenon has been known for fifty years but we still do not know how it works. However, in most of the behavioral assays designed to study antifeedants, sugar is added with the deterrent molecule in order to increase the flies' motivation, which would otherwise be very low. These protocols do not allow the distinction between the antifeedant detection by the L2 cell and the potential inhibiting effect of this molecule on sugar detection by the S cell. In this work, we have shown that, at a certain concentration, some molecules inhibited almost completely the detection of sugar while others did not have any effect. The molecules we tested were all alkaloids, which means that, even amongst a chemical family, the inhibiting potency can vary. Many hypotheses exist about the mechanisms underlying sugar inhibition. We decided to study one hypothesis which postulates a lateral interaction between the bitter- and sugar-sensing cells. We used the UAS-Gal4 system to kill selectively the GR66a-expressing L2 cells and we studied the behavioral and electrophysiological response of the flies without bitter-sensing cells. We showed that the death of these cells abolished the response to strychnine but not to sucrose. Moreover, flies lacking the L2 cell still showed sucrose inhibition by strychnine in their labellar sensilla and still avoided strychnine mixed with sucrose in our MultiCAFE assay. Taken together, these results suggest that the inhibition of sugar detection by the S cell in presence of antifeedants is not due to an action of the bitter-sensing cell on the sugar-sensing cell but seems to be due to a direct action of the antifeedants on the S cell. Thus, the two modes of action of the deterrent molecules on the taste receptor cells seem to be independent mechanisms. We can wonder if the decrease in taste sensitivity accompanying habituation comes from a modification of both phenomena or if only one of them is modulated.

In this PhD, the first necessary step to show a correlation between taste sensitivity and the activity of detoxification enzymes in the habituation phenomenon was logically to establish a situation of habituation in the fruit fly. However, we failed to find a protocol eliciting habituation to caffeine in *D. melanogaster*. It is difficult to assess whether this phenomenon does not happen in this species or whether the conditions we tested (concentrations, exposure durations, molecule...) were not optimal. Habituation has never been shown in the fruit fly so no reference situation was available. Moreover, we found that metyrapone, the pharmacological agent we had

planned to use as a P450s inhibitor, had an effect on sugar detection which would interfere with our experiments. Considering that the adjustment of our behavioral assay, the MultiCAFE, had already needed some time and that finding a reference situation of habituation would have required time as well, we decided to go further in the understanding of another puzzling phenomenon, the aforementioned inhibition of sugar detection by some deterrent molecules.

2. Perspectives

In this work, we have described a quantitative multiple-choice assay, called the MultiCAFE. As we said before, there are still some improvements which could be made on this setup. First, we have talked about the low solubility of some molecules in water and we have suggested dipping sugar-filled capillary tubes in a solvent containing different concentrations of the molecule. This solution looks promising but we have to study it thoroughly in order to detect technical problems. Moreover we have to see if the flies can override their aversion and learn that the tubes only contain sugar, which would interfere with the outcome of the experiments. Secondly, we could improve the measurements of the levels of liquid on the pictures taken before and after the two hours of test. Indeed, the ImageJ software can isolate areas of a certain color and we could use a Matlab program to partly automate the analysis of the pictures. Concerning the statistical analysis of the collected data, we can think of another, maybe complementary, way to find differences between dose-response profiles. Indeed, we could fit sigmoidal curves on the data we obtain with the MultiCAFE. Each treatment would then correspond to a sigmoidal function with a particular set of parameters. We could then compare these parameters between treatments. This type of analysis has to be explored in order to see if it can be applied to our data and if it makes the comparison between treatments easier.

On the other hand, we have said that the MultiCAFE opened to new questions, especially about the way flies choose their food among multiple-options. Tinette *et al.* (2004) suggested that flies did not individually check each food source option. There is a cooperative behavior between “primer” flies, exploring the environment, and “followers”, directly moving to the good food source, without sampling the other possibilities. This cooperation is likely to be based on sensory cues. As we suggested earlier, marking might be involved in the feeding choices of the flies. Testing anosmic flies in the MultiCAFE would help answer that question. Moreover, we

could fill the capillary tubes with a sugar mixed with different concentrations of 11-*cis*-vaccenyl acetate and study the short term feeding choice of naïve flies.

Here, we have shown that the inhibition of sugar detection by antifeedant molecules was not due to a lateral interaction of the bitter-sensitive cell. The more likely hypothesis would be a direct action of the aversive molecules on the S cell (Figure 39). New experiments should be considered to precise the mechanisms of this inhibition.

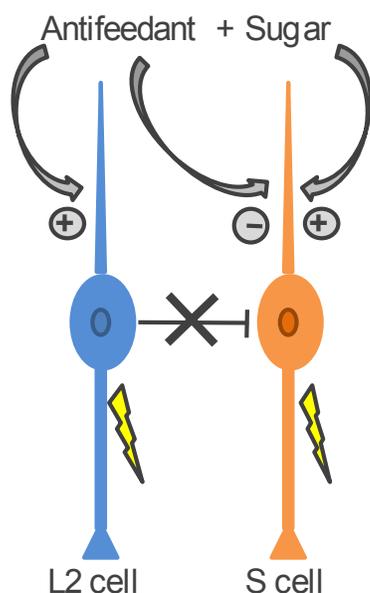


Figure 39. New hypothesis on sugar detection inhibition by antifeedants.

This phenomenon should be due to a direct effect of the deterrent molecule on the S cell rather than to a lateral inhibition by the L2 cell.

First, we can wonder if this inhibition depends on the sugar or if the presence of the antifeedant causes a general inhibition of the S cell whichever sugar is mixed with the bitter molecule. Building electrophysiological dose-response curves using different sugars mixed with quinine or strychnine could help answer that question. If the S cell inhibition does not depend on the sugar, then it would mean that the binding site for the bitter molecules on the S cell should be distinct from the sugar binding site. If the S cell inhibition does depend on the sugar, then the more likely explanation would be that the antifeedants bind to a site which is somewhat related to the binding site of the molecules concerned by the inhibition.

The UAS-Gal4 system could be used to express the capsaicin receptor VR1 or channelrhodopsin 2 in the S cell. The mammalian vanilloid receptor (VR1) is a cationic channel

belonging to the transient receptor potential (TRP) superfamily and activated in response to the hot chili pepper ingredient capsaicin (Caterina *et al.*, 1997), a neutral molecule for fruit flies. Marella *et al.* (2006) used the UAS-Gal4 system to express VR1 in sugar- or bitter-sensing cells in *D. melanogaster*. They performed binary feeding tests and showed that, according to the cell expressing VR1, the flies are attracted or deterred by capsaicin. A possible experiment would be to generate flies expressing VR1 in the S cells and stimulate their labellar sensilla with capsaicin and different concentrations of strychnine to look for an inhibition of the capsaicin response by strychnine. On the other hand, channelrhodopsin2 (ChR2) is a seven transmembrane domain protein responding to light stimulation by opening an internal cation channel (Nagel *et al.*, 2003). Using UAS-Gal4 strains, Zhang *et al.* (2007) expressed ChR2 in sugar-sensing cells of the fruit fly taste sensilla and induced proboscis extension upon stimulation with blue light. We could record the electrophysiological response of these flies to blue light while bathing the sensillum with different concentrations of strychnine. As in the aforementioned experiment with different sugars, knowing if strychnine still has an inhibiting effect on the response of the S cell to capsaicin or blue light would help us determine if strychnine binds to a site which is linked to the binding site of the inhibited molecule or not.

Many genetic tools are available in *D. melanogaster* but pharmacological agents can also be a way to get more information on sugar inhibition. Using drugs to inhibit specific elements of the transduction pathways can be used to unravel the transduction mechanisms involved in sugar detection inhibition by antifeedants. However, the transduction mechanisms involved in taste are still mostly unknown and this pharmacological approach may seem a little hazardous at this time.

The 8 receptors belonging to the sugar receptors family are partly co-expressed (Dahanukar *et al.*, 2007; Jiao *et al.*, 2007) and the function of GR64b-e and GR61a is still unknown. The expression of GR64a coupled with GR5a or GR64a is not sufficient to confer the ability to respond to sugars to otherwise sugar-insensitive cells (Jiao *et al.*, 2008). Thus, GR64b-e and GR61a are likely to form multimers with GR5a, GR64a and GR64f and to contribute to sugar detection. However, they may also be involved in sugar detection inhibition by antifeedants. Flies which *Gr61a* or *Gr64b-e* genes have been impaired could be tested with the MultiCAFE and with single sensillum recordings to check for a potential involvement of these receptors in sugar detection inhibition.

In mice, TRPM5 is a Ca^{2+} - and voltage-activated non-selective cation channel which belongs to the TRP family and is involved in the transduction of sweet, bitter and umami tastes. TRPM5 is inhibited by quinine and by quinidine, the stereoisomer of quinine, but not by denatonium benzoate. The effect of quinine or quinidine on TRPM5 leads to the inhibition of TRPM5-dependent responses of single sweet-sensitive fibers to sucrose (Talavera *et al.*, 2008). This direct effect of quinine on the sugar-responsive cells in mice is in favor of our findings and tends to suggest that a similar phenomenon is possible in *D. melanogaster*. It would be interesting to look for an equivalent channel in the fruit fly and test if this channel is also involved in sugar detection inhibition.

As we said before, a reciprocal inhibition of the L2 cell by sugars may exist. Moreover, this inhibition could happen when sugars are mixed with some antifeedants but not others. Complementary experiments are necessary to confirm these observations. First, we could build electrophysiological dose-response curves with a constant concentration of strychnine or lobeline and increasing concentrations of sugar, for example, to look for a dose-dependent effect. If this bitter-sensing cells inhibition by sugars is confirmed, then *Gr5a-DTI* flies could be generated to see if the S cell is involved in this phenomenon or not, as we did here for the opposite inhibition.

In this work, we failed to set up a situation of habituation with caffeine. As we said earlier, the molecule and the exposure durations may not have been optimal. It might be interesting to try other deterrent compounds and maybe shorter or longer exposure durations. Moreover, we found that metyrapone had an inhibitory effect on sugar detection. This prevented us from using it further as an inhibitor of the P450s enzymes. We would have to find other drugs to modulate the activity of these detoxification enzymes, like the inductor phenobarbital (Danielson *et al.*, 1998; Le Goff *et al.*, 2006; Sun *et al.*, 2006) or the inhibitor piperonyl butoxide (Frank and Fogleman, 1992; Snyder and Glendinning, 1996). As for metyrapone, the potential effect of these drugs on sugar detection would have to be studied. Another way to modulate the expression of the P450s enzymes would be to use genetical tools, like the UAS-Gal4 system. However, this implies to collect preliminary results, with RNA microarrays for example, in order to select candidates which expression is modulated by the exposure to antifeedants. Then, we

could selectively over- or under-express them and see if we obtain a change in the consumption of deterrent compounds.

Akhtar *et al.* (2003) showed that aversive stimuli like cold, CO₂ or deterrent compounds could cause dishabituation. The 22-hour starvation before the experiments or the transfer of the flies with a pooter may be considered as stressful situations and might elicit a reset of the flies' feeding behavior. In other words, the exposure to caffeine may have caused habituation in our fruit flies but some stress posterior to the exposure period might have counteracted the effect of caffeine. We could try to reduce starvation before the experiment and transfer the flies in a more gentle way. Decreasing the hunger state of the flies before the experiments might lead to lower intakes but to higher discrepancies between the consumption of exposed and naïve flies.

As we were unable to elicit habituation to antifeedants, we can also wonder if habituation is as widespread in adults as in larvae. Indeed, if we look at the habituation studies mentioned earlier, we note that most of them were conducted on larvae and not on adults. The larvae are less mobile than the adults and may thus have a more limited number of food options. They might need a higher feeding plasticity than adults to cope with this situation. It would be interesting to adapt our habituation protocol and see if this phenomenon is more likely to be elicited in fruit fly larvae. On the other hand, adult *D. melanogaster* can habituate to sucrose in PER and to other stimuli in various sensory modalities (see review in Engel and Wu, 2009). It is difficult to imagine why this phenomenon would not occur in adults for the consumption of deterrent compounds.

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Résumé

Le goût représente le dernier point de contrôle avant l'ingestion de nourriture. Ceci est particulièrement vrai dans le cas des herbivores car les plantes contiennent de nombreux composés répulsifs et potentiellement toxiques. Chez *Drosophila melanogaster*, la gustation fait intervenir des structures en forme de poils appelées sensilles et localisées sur les pièces buccales, les tarsi, l'ovipositeur et le bord des ailes. A l'extrémité de la sensille, un pore permet l'entrée des molécules dans le conduit sensillaire contenant les dendrites des neurones chimiorécepteurs. Chaque sensille renferme un neurone mécanorécepteur et généralement quatre neurones chimiorécepteurs classés selon le type de goût auxquels ils répondent. Dans ce travail, nous avons étudié principalement les cellules qui détectent les sucres (type « S ») et les cellules qui détectent les substances aversives (type « L2 »).

Nous avons développé un test comportemental pour mesurer les préférences alimentaires chez *D. melanogaster*. Ce test repose sur la mesure quantitative de la consommation de solutions proposées à des groupes de mouches dans plusieurs capillaires de verre, dispositif que nous avons appelé MultiCAFE (MULTIple CAPillary Feeder). La multiplicité des choix fournis aux mouches ne semble pas réduire leur capacité de discrimination, la sensibilité du test étant la même que celui-ci soit préparé comme un test de non-choix, un test binaire ou un test à choix multiples. Ce nouveau test nous a permis de classer huit alcaloïdes selon leur pouvoir antiappétant, évalué selon deux dimensions : la capacité de discrimination et la consommation totale des mouches.

Nous avons étudié la perception de ces alcaloïdes au niveau des sensilles gustatives du proboscis de ces mouches, en utilisant des enregistrements électrophysiologiques extracellulaires. Les composés aversifs ont deux modes d'action sur les sensilles gustatives : ils activent la cellule L2 mais peuvent également inhiber la cellule S s'ils sont mélangés à des sucres. Nous avons mis en évidence que l'inhibition périphérique de la détection des sucres était spécifique : à une concentration donnée, la strychnine et la lobeline inhibent presque complètement la détection du saccharose alors que la caféine et la nicotine n'ont aucun effet. Les mécanismes qui sous-tendent ce phénomène sont toujours inconnus. Nous avons testé l'hypothèse d'une interaction latérale entre les cellules L2 et S. En utilisant le système UAS-Gal4 pour tuer sélectivement les cellules L2, nous avons montré que la détection réduite du saccharose par la cellule sensible aux sucres en présence de strychnine n'était pas due à une inhibition latérale causée par la cellule sensible à l'amer. De ce fait, l'hypothèse la plus plausible est que l'inhibition de la détection des sucres par les antiappétants se déroule directement au niveau du neurone gustatif sensible au sucre. D'autres expériences sont nécessaires pour préciser si le mécanisme impliqué dans cette inhibition est présent au niveau du site de fixation du sucre ou pas.

Summary

Taste represents the last checkpoint before food ingestion. This is particularly true for herbivores as plants contain many deterrent, potentially toxic, compounds. In *Drosophila melanogaster*, taste involves hair-like structures called sensilla and located on the mouthparts, the tarsi, the ovipositor and the wings margins. At the tip of the sensilla, a pore allows the entry of the molecules in the hair shaft containing the dendrites of the chemosensory neurons. Each sensillum houses one mechanoreceptor neuron and two to four chemosensory neurons which are classified according to the taste quality they respond to. In this work, we have focused on the S cell, sensitive to sugars, and the L2 cell, which detects aversive molecules in many sensilla.

We have developed a behavioral assay to measure feeding preferences in *D. melanogaster*. This assay, called MultiCAFE (MULTIple CApillary Feeder), quantitatively measures the consumption of solutions provided to groups of flies in several glass capillary tubes. The multiplicity of choices provided to the flies does not seem to reduce their discrimination ability as the sensitivity of the assay was found to be the same whether it was set up as a no-choice, two-choice or multiple-choice assay. This test allowed us to rank various alkaloids according to their antifeedant potency, evaluated according to two dimensions: the discrimination abilities of the flies and their total consumption.

We have studied the perception of these alkaloids in the taste sensilla of the proboscis, using extracellular electrophysiological recordings. Deterrent compounds have two modes of action on the taste sensilla: they activate the L2 cell but also inhibit the S cell if they are presented in mixture with sugars. We highlighted the specificity of this sugar detection inhibition, showing that, at a given concentration, strychnine and lobeline almost completely inhibited sucrose detection whether caffeine and nicotine did not have any effect. The mechanisms underlying this phenomenon are still unknown. We tested the hypothesis of a lateral interaction between the L2 and the S cell. Using the UAS-Gal4 system to kill selectively the L2 cells, we showed that the decrease in sucrose detection by the sugar-sensing cell in presence of strychnine is not due to a lateral inhibition caused by the bitter-sensing neuron. Thus, the most likely hypothesis is that sugar detection inhibition by antifeedants directly takes place at the level of the sugar-sensing neuron. More experiments are needed in order to precise if the mechanisms involved in this phenomenon occur at the sugar receptor site or not.