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Consumption of Bitter Alkaloids in *Drosophila melanogaster* in Multiple-Choice Test Conditions

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Abstract

Drosophila melanogaster adapt their food consumption to their internal needs and avoid ingesting noxious molecules. Defects in the genes involved in these decisions induce behavioral alterations that are usually screened by monitoring flies feeding in 2-choice or in no-choice situations. Here, we introduce a new behavioral test in which groups of flies are given access to 6 capillary feeders (MultiCAFE) containing fructose mixed with a serial dilution of a test substance. Using quinine, we first showed that fly density, distance between capillaries, and order of presentation have a minor impact on the discrimination performances of the flies. Fly discrimination was also only marginally affected by the type of test (no-choice, binary, or multiple-choice). Interestingly, the feeding reduction was well correlated with a reduction of the firing elicited by the mixture in sugar-sensitive gustatory receptor neurons, suggesting that several mechanisms concur to allow flies to make their choices. In addition to quinine, flies exhibited marked dose-dependent aversions to the consumption of berberine, caffeine, lobeline, nicotine, papaverine, strychnine, and theophylline, which all taste bitter to humans. Thus, despite of the multiplicity of choices available, flies consistently avoid alkaloids mixed with a sugar solution, and their choices are strongly dependent on their taste system. The MultiCAFE assay represents an interesting alternative to other feeding tests, in that it allows monitoring of the absolute consumption while also requiring less flies and time to run than other assays.

Key words: behavior, electrophysiology, food choice, fructose, fruit flies

Introduction

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). In *Drosophila* adults, contact chemoreception is mediated through hair-like structures, called sensilla, located on the mouthparts, the legs, the wings margin, and the ovipositor. The contact chemosensory sensilla located on the mouthparts, that is, on the labellum or proboscis, directly influence feeding activities and are designated as taste sensilla. All taste sensilla have a pore at their tip that allows chemicals to penetrate the hair shaft and contact the dendrites of gustatory receptor neurons (GRNs). The labellar sensilla are classified into 3 types according to their length (l, long; s, small; and i, intermediate) (Shanbhag et al. 2001). l- and s-type sensilla house 4 GNRs 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 (Hiroi et al. 2002; Lee et al. 2009). i-type sensilla only contain 2 GRNs, 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).

A family of 68 candidate gustatory receptors (GRs) has been identified (Clyne et al. 2000; Dunipace et al. 2001; Scott et al. 2001). Although a few of them are known to be involved in sugar or pheromone perception, many could be involved in the detection of aversive molecules (Amrein and Thorne 2005). A family of phylogenetically linked receptor genes is expressed in the sugar-sensing GRNs: *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 coreceptor (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).

Several studies show that *D. melanogaster* is 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 are detected by bitter-sensing GRNs and they elicit 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 which involves GR66a and GR93a, possibly as coreceptors at least for the detection of caffeine (Lee et al. 2009). However, other elements are probably involved since the misexpression of these 2 receptors in sugar-sensing cells is not enough to confer them the capability to detect caffeine (Moon et al. 2006; Lee et al. 2009). Recently, *Gr33a*, which is phylogenetically the closest *Gr* gene from *Gr66a*, has been shown to also contribute to the sensitivity of bitter-sensitive cells toward several alkaloids (Moon et al. 2009). However, if *Gr33a* is likely to act as a coreceptor to the other 2 receptors, again, the misexpression of *Gr66a*, *Gr93a*, and *Gr33a* into sugar-sensitive cells is not sufficient to allow these cells to respond to bitter substances (Moon et al. 2009).

Apart from electrophysiological recordings, all these studies have relied on behavioral tests comparing the feeding preferences of mutant and control flies. So far, all existing procedures test the discrimination abilities of the flies but do not take into account how much is consumed. The simplest approach consists in recording how many flies wander on a treated surface as compared with a control surface (Marella et al. 2006) but this behavior is only indirectly related to feeding. The most commonly used test consists in allowing flies to feed on 2 food substrates including different food dyes (Tanimura et al. 1982). After exposure to the food, their abdomen color is checked (red, blue, or purple when they fed on both sources) and a preference index is computed. 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.

Given the limitations of these tests, we propose another approach to evaluate flies selectivity and absolute consumption. In rats and mice, "self-service bottles" are commonly used (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-μL 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-μL microcapillary tubes. With this system, called Capillary Feeder (CAFE), they analyzed the feeding behavior of flies, the influence of population density or humidity and the impact of ethanol or paraquat on food intake. 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 2-choice assay on *D. melanogaster* to study the regulation of feeding by peripheral clocks (Xu et al. 2008) or how different protein–carbohydrate ratios affect life span and fecundity (Lee et al. 2008).

In this paper, we evaluate the feeding preferences of flies provided access to multiple capillary feeders (MultiCAFE). Groups of flies were provided access to series of 6 capillary tubes filled with solutions containing different concentrations of an antifeedant. This approach gives the possibility to build dose-response profiles directly. We examined the effect of different parameters on the sensitivity of the setup, and we compared curves obtained with the MultiCAFE used as a multiple-choice, no-choice, or 2-choice test. Secondly, we tested the correlation between this feeding test and electrophysiological data recorded from peripheral taste sensilla. Then, we used MultiCAFE experiments to compare the behavioral effect of 8 alkaloids and discuss the differences in antifeedant potency of these molecules. Finally, we use the MultiCAFE to observe the response to caffeine of flies which have been reported to have a defect in the detection of this molecule.

Materials and methods

Chemicals

Lobeline, papaverine, quinine, nicotine, berberine, strychnine, caffeine, and theophylline were provided by Sigma-Aldrich. All chemicals were dissolved into 35 mM fructose (Sigma-Aldrich) supplemented with brilliant blue (0.125 mg/mL, FCF [C37H3409SNa], Tokyo Kasei Co.). Solutions were prepared in advance and stored at –20 °C until use.

Flies

Stocks of *D. melanogaster* (Canton-S, w^{1118} and $\Delta Gr66a^{ex83}$) were maintained on a standard cornmeal agar medium, at 25 °C and 80% humidity, on a 12:12 light-dark cycle.

MultiCAFE

Emerged flies (~1 day old) were transferred to a freshly prepared food medium for 2–3 days and maintained in a rearing chamber at 25 °C. The flies were first sexed (after numbing them on cold ice) and were then transferred to plastic tubes provided with humidified filter paper and starved for 20–22 h. Just before the experiment, these flies were transferred into experimental vials (23.5 diameter × 40 mm, SARSTEDT) by groups of 10–60 flies depending on the experiment. All experiments were performed at 25 °C in complete darkness.

Experimental vials were closed by a plug (28.5 mm Buzz-Plugs, Fisherbrand), cut to 0.8-cm height, and sliced into 2 halves (Figure 1, left). On one half of this modified plug, we placed a row of six 5 μL microcapillary tubes (Hirschmann Laborgeräte) 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. 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. 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. All experiments were performed at the beginning of the afternoon to prevent any effect of the circadian rhythm.

The comparison between the curves for a no-choice, 2-choice, and multiple-choice assay was done with a slightly modified setup. The 6 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 × 76 × 15 mm, Caubère) with repositionable adhesive pads (Patafix, UHU) (Figure 1, right). Flies were transferred into the box without anesthesia. 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 2-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). The multiple-choice test (n = 20, 20 unsexed flies per box) was conducted as in the vials.

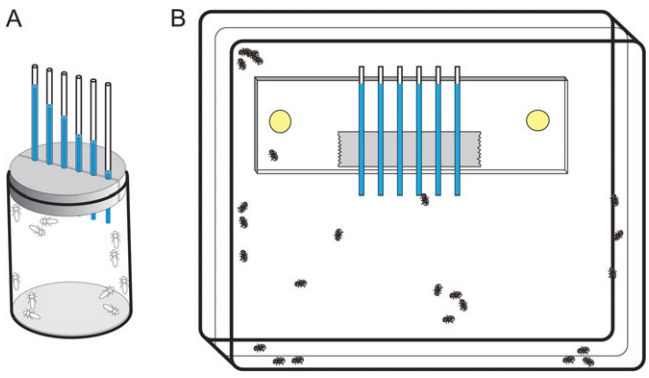

Figure 1 Schematic representation of the MultiCAFE assay. Flies are inserted into a vial (A) or a box (B) and provided with 6 capillary tubes filled with different solutions. After 2 h, the level of liquid in the capillaries is measured, and evaporation is subtracted to calculate the actual consumption of the flies. This figure appears in color in the online version of *Chemical Senses*.

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 from this value the amount of liquid evaporated within the control vial without flies. To be able to compare curves obtained in different conditions, we normalized the responses by expressing the consumption per fly and per hour.

Experimental conditions tested

Four series of experiments were performed. First, we evaluated different experimental conditions using quinine as a test stimulus in order to establish a working protocol. These conditions were: fly density (n = 10, 20, 40, or 60 flies per tube), an ascending versus a random order of the capillaries, and distance between the capillaries (0, 1, 3 mm). Each condition was tested on 10 groups of flies or each sex. Because the major differences between the various conditions were found in females, we only present the results for this sex (the results for males are presented in Supplementary Figure S1). Secondly, we compared the sensitivity of the test used as a no-choice, 2-choice, or multiple-choice assay. Then, we compared the biological activity of different alkaloids with the MultiCAFE, using lobeline, papaverine, quinine, nicotine, berberine, strychnine, caffeine, and theophylline. Control tests with fructose were run in parallel. For each condition, we performed 10 repetitions for each sex. Lastly, we compared the responses of w^{1118} and *ΔGr66a* mutant flies with caffeine. We repeated the experiment 10 times, using males exclusively, as males with a w^{1118} background seem to have a higher consumption than females (Supplementary Figure S4).

Electrophysiological recordings

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. Individual taste sensilla were stimulated by covering their tip with an electrode containing an electrolyte (1 mM KCl) and a stimulus during 2 s (Hodgson et al. 1955). To avoid adaptation, consecutive stimulations were applied at least 1 min apart. We recorded signals from L5 and I9 sensilla (nomenclature described in Hiroi et al. 2002) with binary mixtures of fructose and quinine at the same

concentrations as in the MultiCAFE experiment. Each experiment started with the presentation of 35 mM fructose. Then, 5 concentrations of quinine (0.001, 0.01, 0.1, 1, or 10 mM), mixed with fructose 35 mM, were presented in ascending order. Finally, the sensillum was stimulated again with 35 mM fructose. Only the sensilla responding to the first and last stimulation were included in the analysis.

The recording electrode was connected to a preamplifier (gain = ×10; TastePROBE DTP-02, Syntech) (Marion-Poll and van der Pers 1996), and electric signals were further amplified and filtered by a second amplifier (CyberAmp 320, Axon Instrument, Inc., gain = ×100, eight-order Bessel pass-band filter = 1–2800 Hz). These signals were digitized (DT9803, Data Translation; 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.

Data analyses

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, and sex) (Roa 1992; Manly 1993). We ran a descriptive multivariate analysis to explore the relationships between variables and then an inferential statistical analysis for the suggested model.

First, to detect patterns of association of variables and to eliminate nonlinear correlations that might exist, we calculated analytically simple linear correlation matrices (Pearson correlation) and we built Scatter Plots Matrices. Secondly, we ran a principal components analysis in order to confirm correlations between variables and to study the association with the various classification variables (e.g., substance, sex, and 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. 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.

Results

Detection of quinine concentration and influence of fly density

In order to establish if flies could distinguish between different concentrations of quinine and the impact of fly density on MultiCAFE tests, we compared their responses with a series of dilutions of quinine using densities of 10, 20, 40, or 60 flies. Each test condition (density × sex) was replicated 10 times.

There is an effect of the density on the individual consumption both in males ($P = 0.0081$, MANOVA, Supplementary Figure S1A) and in females ($P = 0.0011$, MANOVA, Figure 2A). The females reduce their uptake with increasing density of flies in the vial. The dose-response curves look very similar across all density conditions, with 50% of the inhibition observed between 0.01 and 1 mM quinine. The major impact

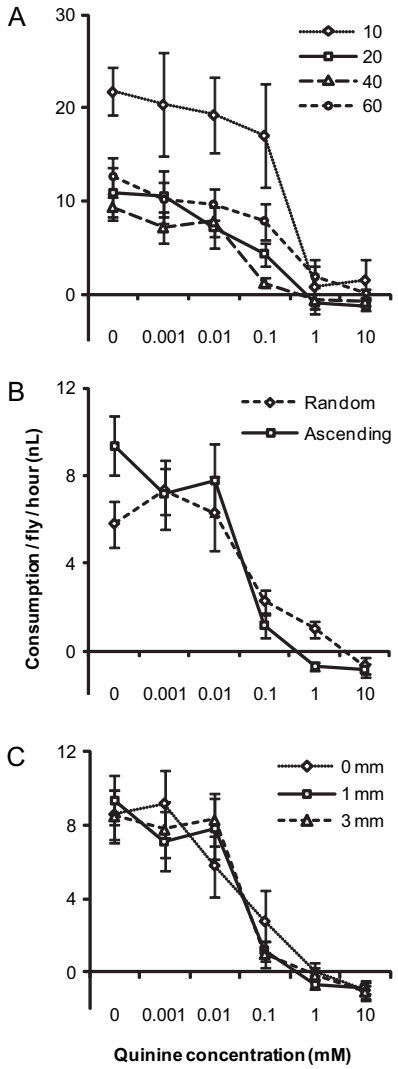

Figure 2 Dose-response curves for quinine with different varying parameters. Males and females were tested separately and only the data for females are presented here. The concentrations, mixed with fructose 35 mM and a blue dye, were presented simultaneously to the flies in 6 microcapillary tubes. Consumption for each concentration was measured at the end of the test session. Comparison of the response to quinine according to **(A)** different densities of flies in the test vials (10, 20, 40, or 60 flies), **(B)** the order of the concentrations in the setup (randomized or ascending order), **(C)** the spacing between the microcapillary tubes in the vials (0, 1, or 3 mm). $n = 10$ for each curve. Error bars represent standard error of the mean.

of density is observed with 10 flies per tube, with a higher consumption per fly and an increased variation across replicates compared with other densities. Conversely, with 60 flies, we observed a lower variability but the consumption decreased and the dose-response curve shifted to the right by about a factor of 10 and show a slight change in its shape. The results for males are quite similar but the difference of consumption for a density of 10 flies is less marked. 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.

Influence of the arrangement of the series of concentration of quinine

We then assessed if the order of presentation of the capillaries had an impact on the dose-response curves, using groups of 40 flies and 10 replicates per condition and per sex. Two conditions were tested: 1) capillaries disposed in a row of increasing concentrations and 2) capillaries disposed in random order, obtained using the random function as a macro under Excel. No difference was observed between the curves for males ($P = 0.1843$, MANOVA, Supplementary Figure S1B).

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 2B). The 2 quinine dose-response curves look very alike though the shape of the curves is slightly different. As in the previous experiment, variability increased when the concentration of quinine was low. Because the effect of arranging the concentration in series or randomly seemed quite modest, we used capillaries arranged in serial order of increasing concentrations in the rest of our experiments.

Effect of the spacing of the capillary tubes

In order to find the best experimental conditions, we next tested if the spacing between the capillary tubes affected the responses to quinine. Indeed, when the capillaries are close to each other, we observed that flies can walk from one tube to the other and, thus, simultaneously sample different solutions with their tarsi. On the other hand, if capillaries touch each other, lack of space and competition may happen. We designed 3 conditions: capillaries touching each other, spaced by 1 mm, or by 3 mm. Each condition (distance × sex) was tested 10 times using groups of 40 flies. We did not find any difference between the spacing conditions either for males ($P = 0.3779$, MANOVA, Supplementary Figure S1B) or for females ($P = 0.2179$, MANOVA, Figure 2C). In fact, the 3 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. Regarding these results and for practical reasons, we chose to use a distance of 1 mm between the capillaries in the remaining experiments.

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 3, we observe that the standard deviation reaches a plateau between 10 and 15 repetitions. With a small number of repetitions, the graph shows that the estimation of variability is far from the target. As we increase the number of repetitions, we reach a better estimation of the actual variability until a point when no more information is added.

Comparison of the MultiCAFE used as a no-choice, 2-choice, or multiple-choice test

To assess the sensitivity of the MultiCAFE, we built a dose-response curve for quinine using the test as a no-choice or

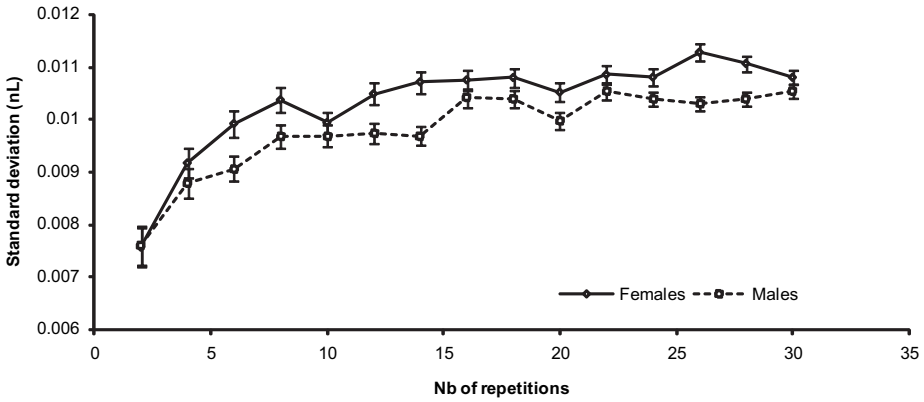

Figure 3 Evolution of the estimation of the variability according to the number of repetitions. We used the 44 repetitions performed with the fructose control, in the alkaloids experiment described in Figure 8, and randomly selected subsets of these data to estimate the variability. $n = 500$ samples for each data point.

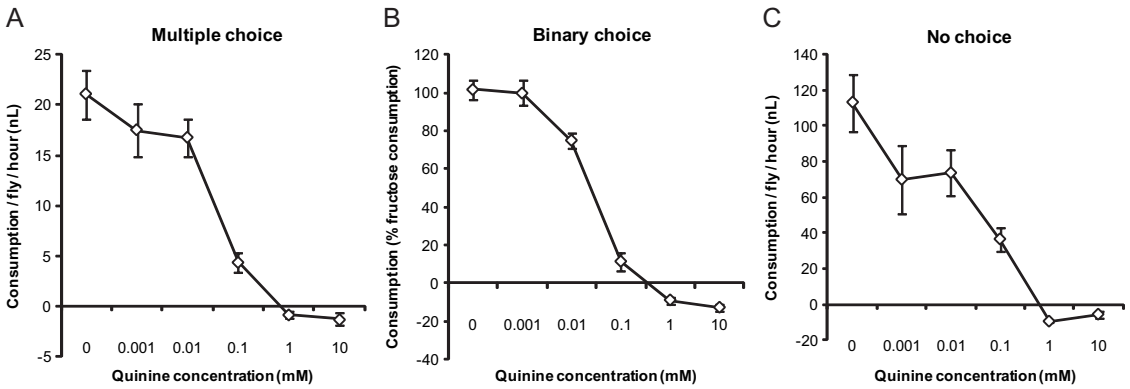

Figure 4 Comparison between the MultiCAFE used as a multiple-choice **(A)**, 2-choice **(B)**, or no-choice **(C)** assay. For the no-choice assay, the 6 capillary tubes were filled with 35 mM fructose and one of the tested quinine concentrations (0, 0.001, 0.01, 0.1, 1, or 10 mM). For the 2-choice assay, 3 capillaries containing 35 mM fructose mixed with one concentration of quinine and 3 capillaries containing fructose alone were alternated in the vials. The consumption in the quinine-containing capillaries is expressed in percentage of the consumption in the fructose capillaries of the same experiment.

a 2-choice assay, in order to compare the results with the multiple-choice curve. For the no-choice assay, the 6 capillaries were filled with the same solution: the blue dye, fructose, and one of the 6 concentrations of quinine. For the 2-choice, we alternated 3 microcapillaries filled with fructose and quinine at the tested concentration and 3 microcapillaries with fructose alone. In this case, we expressed the consumption in the capillaries containing quinine as a percentage of the consumption in the capillaries containing only fructose. The absolute consumptions are given in Supplementary Figure S2.

We estimated graphically the half maximal effective concentration (EC_{50}) of the curves on Figure 4 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 3 curves and was around 0.02–0.03 mM.

Correlation between the electrophysiological and the behavioral responses

Lastly, we compared the dose-response curves obtained with the MultiCAFE used as a no-choice, 2-choice, or multiple-choice test 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 2 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, whereas L5 sensilla house 4 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 decreases as the concentration of quinine increases (Figure 5). This spiking inhibition

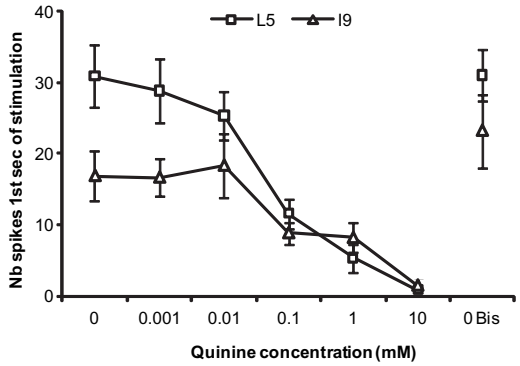

Figure 5 Electrophysiological dose-response curves obtained for fructose 35 mM mixed with different concentrations of quinine. The recordings were made on the L5 and I9 sensilla. The different concentrations of quinine were tested in ascending order and another recording with fructose alone was done 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 taken into account.

induced by quinine is fully reversible because 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 poststimulus histograms of the responses using 100-ms bins (Figure 6). These data show that quinine inhibits both the phasic part of the responses (first 200 ms) as well as the tonic responses (after 400 ms). Unexpectedly, we did not record a clear increase of firing at high doses of quinine in I9 sensilla as expected because 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-sensitive cells to the responses observed.

In order to estimate if the electrophysiological responses can be used to predict the behavioral activity, we plotted the electrophysiological responses across behavioral responses

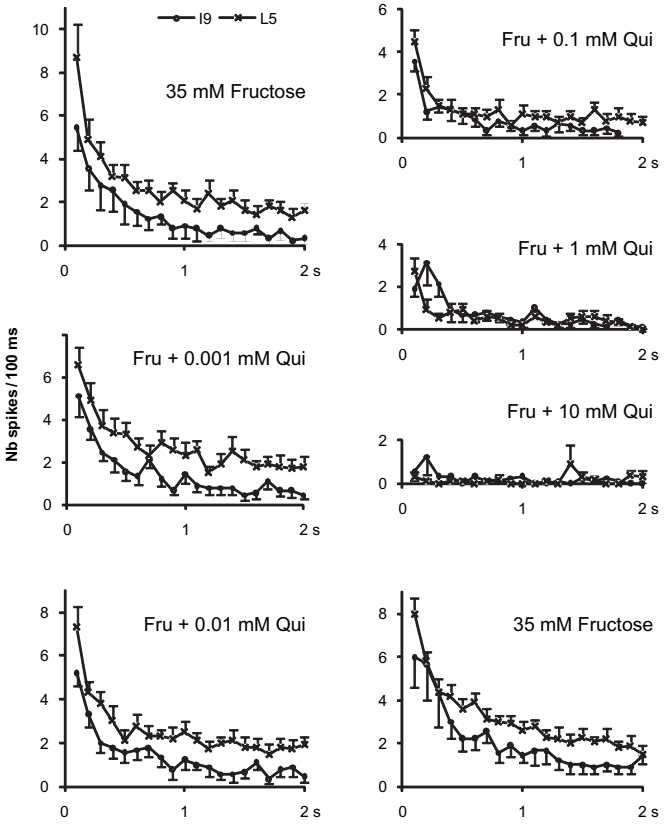

Figure 6 Number of spikes per 100 ms over 2 s of stimulation with a mixture of fructose 35 mM and different concentrations of quinine. For details, see Figure 5. These data indicate that quinine inhibits both the phasic part of the responses (first 200 ms) as well as the tonic responses (after 400 ms).

obtained with the same doses, in the MultiCAFE used as a no-choice, 2-choice, or multiple-choice test (Figure 7). As these data were not obtained on the same individuals, we compared the average consumption obtained in the 3 sets 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, that is, the response for 35 mM fructose. The 3 behavioral sets of results are highly linearly correlated with the electrophysiological data ($R > 0.95$ for the 3 regressions). The regression curves are very similar for the 3 types of assays. We note that these curves do not cross the y axis at 0 but at about 20% of the maximal response. This may represent a threshold under which the peripheral response does not induce any feeding response.

Dose-consumption profile for 8 alkaloids

In this experiment, we tested 8 common alkaloids: berberine, caffeine, lobeline, nicotine, papaverine, quinine, strychnine, and theophylline. Each experimental condition was repeated 10 times per sex, using groups of 20 flies. Data from males and females are given in Supplementary Figure S3. They 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).

Each of these chemicals was found to inhibit feeding according to the dose (Figure 8A). They differ however by their threshold of activity. This activity was estimated graphically by measuring the EC_{50} from the curves. These values represent the concentration of antifeedant leading to a consumption equal to 50% of the consumption in the capillary

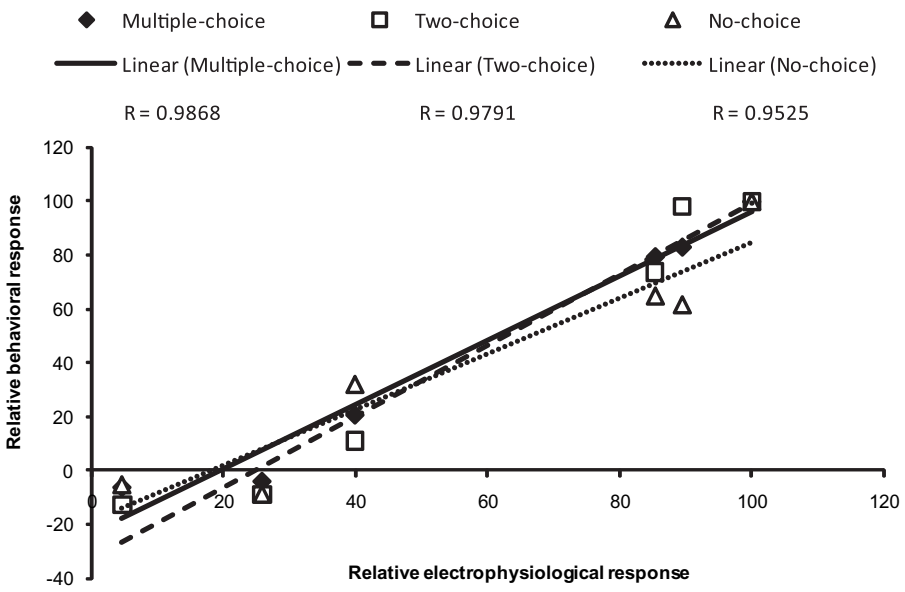

Figure 7 Linear correlation between the electrophysiological response (number of spikes during the first second of stimulation, average of L5 and I9) and the behavioral response (consumption in the MultiCAFE, average of the data obtained with the no-choice, 2-choice, and multiple-choice assay) for quinine. The responses are expressed in percentage of the maximal response (for fructose alone).

containing fructose only. According to EC_{50}, the biological activity of this series of alkaloids is as follow: strychnine > lobeline > berberine > theophylline > quinine > caffeine > papaverine > nicotine (Table 1). Intriguingly, we observe an increase in consumption for papaverine 0.1 mM.

If we look at the total consumption for each substance, we can see that "compensative" feeding did not happen for all the substances (Figure 8B). 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.

Responses of a *ΔGr66a* mutant to caffeine with the MultiCAFE

In this last experiment, we tested the response of a *ΔGr66a* strain, which has been reported to be deficient in caffeine detection (Moon et al. 2006). We compared the response of these flies with caffeine and fructose with the response of w^{1118} flies, as the *ΔGr66a* strain was made from a w^{1118} background. We used only males as preliminary experiments showed that in w^{1118} flies, males had a higher consumption than females (Supplementary Figure S4).

We found that *ΔGr66a* flies consumed less overall than w^{1118} flies (Figure 9, $P = 0.0009$, 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 *ΔGr66a* 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 are significantly different between the 2 strains, which seems to confirm the fructose detection deficiencies or hunger defects in *ΔGr66a* flies ($P < 0.05$, Hotelling). For the 4 curves, we then compared each combination of concentrations. The w^{1118} strain shows a clear caffeine dose-response curve with a good discrimination of caffeine at high concentrations, the highest dose tested being different from all the others ($P < 0.0001$, MANOVA using the Bonferroni criterion). On the other hand, the caffeine dose-response curve for *ΔGr66a* flies is much flatter and there is no difference between the concentrations. We did not find any difference between concentrations in the 2 fructose dose-response curves.

Table 1 EC_{50} of the 8 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

Discussion

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 gives the possibility to build dose-response curves and to screen for the bioactivity of molecules quickly. This multiple-choice test was adapted from the CAFE assay (Ja et al. 2007). 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). We also showed that the EC_{50} drawn from the curves was similar whether the test was used as a no-choice, 2-choice, or multiple-choice assay. Then, we showed that the feeding behavior monitored with the MultiCAFE is highly correlated with the inhibition of the response of taste neurons to sugar. We evaluated the activity of 8 alkaloids using the MultiCAFE to build the corresponding dose-response curves. Finally, we tested a strain previously reported to have deficiencies in caffeine detection (Moon et al. 2006) with the MultiCAFE.

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 *ΔGr66a* 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 9 time less chemicals than the colored wells test (Table 2).

One of the potential limitation of MultiCAFE is that it may be more difficult for flies to discriminate among the different capillary feeders because of the multiplicity of choices available (Prince et al. 2004). The consumption of 2 substances or 2 concentrations can differ greatly whether they are presented alone or simultaneously (Shimada et al. 1987; Akhtar and Isman 2004). In the same way, multiple substances (or concentrations) presented at the same time can be

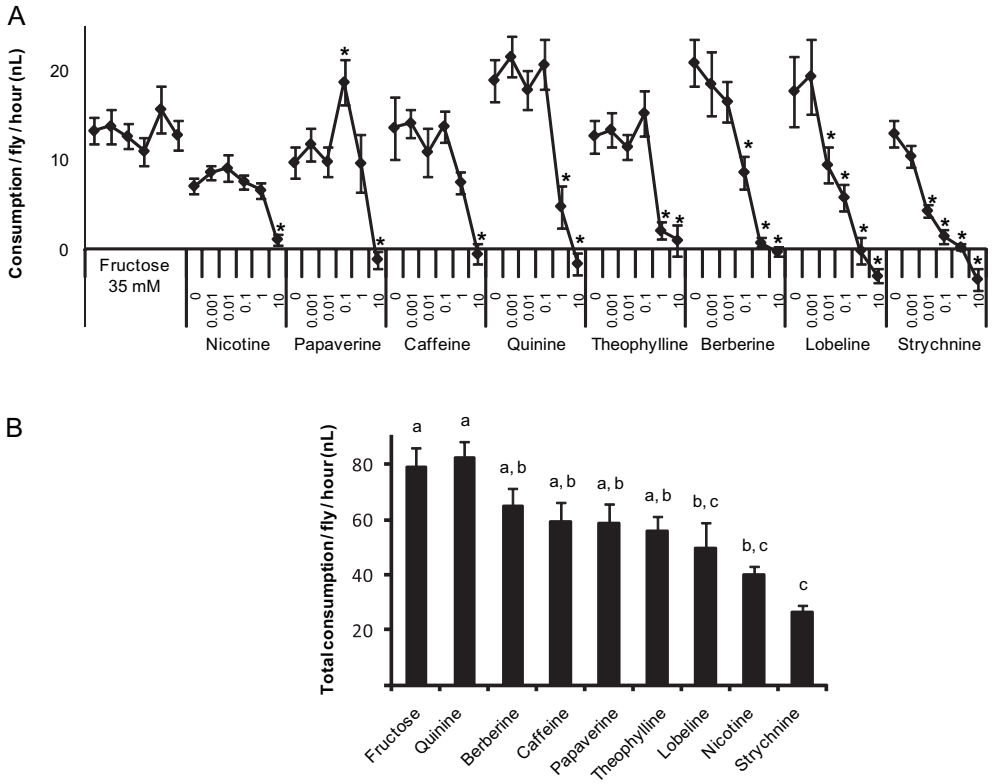

Figure 8 Dose-response curves **(A)** and total consumption **(B)** for fructose, nicotine, caffeine, quinine, papaverine, theophylline, lobeline, strychnine, and berberine. For details, see legend Figure 2. The fructose response corresponds to a control where the 6 capillary tubes are filled with the same solution (fructose 35 mM and the blue dye). Error bars represent standard error of the mean. 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 consumptions, data marked by different letters are significantly different (Bonferroni, $P < 0.05$).

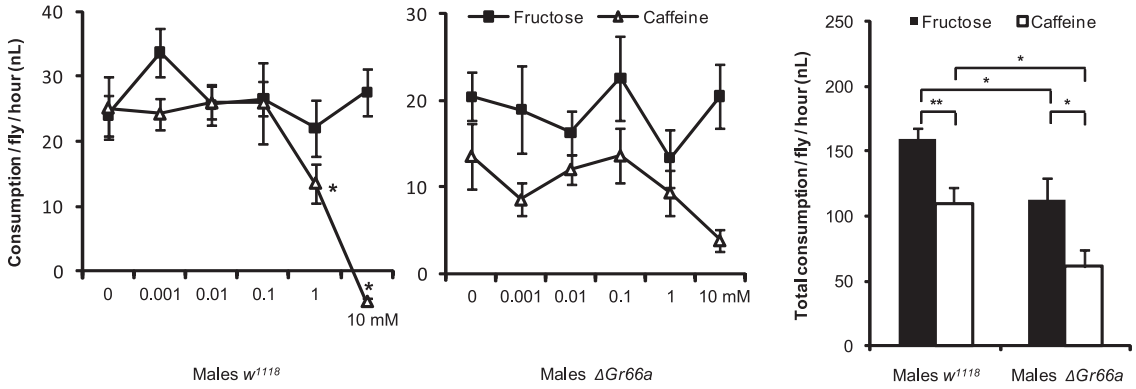

Figure 9 Total consumption and dose-response curve for caffeine and the fructose control tested on *ΔGr66a* and w^{1118} flies. Only males were tested, as preliminary results showed that males of w^{1118} background had a higher consumption than females. On the curves, the asterisks represent concentrations 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 (*$P < 0.05$, **$P < 0.01$).

more difficult to discriminate as compared with 2-choice assays (Raffa et al. 2002). In addition, the aversion toward high concentrations of bitter substances could make the lower concentrations more attractive than they actually are, when compared with a control solution in a binary assay. This could influence the apparent antifeedant potency of a given concentration of a bitter substance in the MultiCAFE. However, the similarity between the curves obtained with the MultiCAFE used as a no-choice, 2-choice, or multiple-choice assay clearly shows that the sensitivity of the 3 kinds

Table 2 Comparison of the need in flies and substance volume between the MultiCAFE used as a multiple-choice test and the test of the blue and red wells, in the case of a dose-response curve of 6 concentrations

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

of experiments may not be so different in our conditions, as the flies discriminate the concentrations as easily in the multiple-choice setup as in simpler preference tests.

The relative consumptions per capillary is, however, 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 test concentrations of a chemical with a control solution but also to compare among test concentrations. This interdependency has to be taken into account when running statistical analyses on results from this test. The approach outlined in this paper takes into account these concerns.

Limiting evaporation in MultiCAFE experiments is particularly important for 3 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 4 or 5 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 compared with tubes which are empty. Thirdly, evaporation may alter the actual concentration of antifeedants experienced by the flies. Because the liquid column is enclosed in 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 is to reduce evaporation as much as possible.

The dose-response curves obtained with the MultiCAFE probably combine the taste discrimination capacities of the flies with memory performances (Motosaka et al. 2007) and a number of social interactions including competition (Dierick and Greenspan 2006; Vrontou et al. 2006) or social facilitation (Shimada et al. 1987; Tinette et al. 2004, 2007). This might explain why the consumption is so irregular between identical capillary feeders (Figure 8: fructose). To assess if density could affect the outcome of the test, we ran the experiment with quinine using 10, 20, 40, or 60 flies. When tested in groups of 10, the flies eat 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 are very similar at the 4 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 occur during the test, such interactions could affect the flies' intake but they do not seem to play a decisive role in feeding choices, under the present conditions.

To our knowledge, this work is the first to examine the bitter potencies of these 10 alkaloids in the same strain of flies. Consequently, it is difficult to compare the bitterness ranking obtained here with other works. However, our ranking is consistent with what has been found in *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. The increase in consumption for papaverine 0.1 mM is difficult to explain. This substance might be appetitive at certain concentrations but more data are needed in order to confirm this observation. We have shown that compensative feeding happened for most of the molecules tested but not for all of them. We can advance some hypotheses. First, lobeline, nicotine, and strychnine 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 GRNs.

Our results on *ΔGr66a* 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 *ΔGr66a* flies consume less than w^{1118} control flies. We suspect that *ΔGr66a* flies may have a hunger deficiency which decreases their uptake whatever the substance. Three hypotheses may arise from this statement. First, the 2 genes flanking *Gr66a* might be involved in uptake regulation. Indeed, the *ΔGr66a* mutant was obtained by the excision of this gene, an excision that also disrupted the 2 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 GR gene, like *ΔGr93a* or *ΔGr33a*, 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.

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, 10^{-3} M KCl may elicit some spikes) (Meunier et al. 2000, 2003; Hiroi et al. 2002, 2004). According to these authors and other studies, bitter substances are detected by L2 cells which express GR66a. However, l-type sensilla are devoid of bitter-sensitive cells, whereas s-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 s-type sensilla or at least, it remained quite inactive because 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). 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.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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