Peripheral interactions between opposing gustatory stimuli in the medial sensillum of the maxilla modify feeding behavior of *Manduca sexta*.

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Abstract

Both chemosensory activity and feeding behavior of Manduca sexta fifth instar larva were measured in response to myo-inositol, a weak feeding stimulant, and rosmarinic acid, a potent feeding deterrent in the nonhost plant, Canna generalis. The activity of chemosensory neurons was identified using a highly effective Bayesian spike classifier which permitted a detailed analysis of both phasic and tonic cell responses. A sensory neuron in the medial styloconicum is strongly activated by inositol, whereas in the same styloconicum a different neuron is strongly activated by rosmarinic acid. In mixtures, rosmarinic acid strongly inhibits the inositol neuron whereas inositol affects the rosmarinic acid neuron mildly and only at some concentrations. Behaviorally, inositol counteracts the deterrenery of rosmarinic acid to a much greater extent than would be predicted from its relatively weak feeding stimulation when presented alone, suggesting a central nervous system process other than simple addition of inputs is responsible. These interactions between the neural and behavioral responses to mixed stimuli are discussed in terms of sensory coding and the associated sensory-behavioral transformation.

Keywords:

feeding behavior; Manduca sexta, chemoreception, sensory code, rosmarinic acid, inositol

Introduction
Phytophagous caterpillars are generally very responsive to deterrent chemicals in their food plants. These phytochemicals can be very important in food selection since they are feeding deterrents at much lower concentrations than they are toxic; see reviews by Frazier (1992); Stadler (1992); Bernays and Chapman (1994); Schoonhoven et al. (1998); Schoonhoven and van Loon (2002). Deterrent-sensitive neurons have been found in all phytophagous caterpillars investigated, and an increase in their spike frequency correlates with a reduction of consumption of foods containing the deterrent Schoonhoven and van Loon (2002). The feeding of larval *Manduca sexta* has been shown to be deterred by rosmarinic acid (RA), the phytochemical proposed by Simmonds et al. (2006) to be responsible for the strong deterrence of *Canna generalis*, a plant on which *M. sexta* refuses to feed even to the point of starvation.

The most important of the chemoreceptors for phytophagous caterpillars are the gustatory sensilla, some of which are located in the maxillary styloconica. For example, larvae of the tobacco hornworm, *M. sexta* will starve to death in preference to eating most non-host plant species, whereas a caterpillar whose major taste organs have been ablated will readily accept these formerly rejected plants; de Boer et al. (1977); de Boer & Hanson (1987). The eight chemosensory neurons of these styloconica in *Manduca sexta* have been identified with a "reference stimulus" that elicits activity from one (and only one) neuron within each styloconicum. Therefore, each of these chemical stimuli is designated as a "reference compound" for a given neuron (e.g., inositol neuron, glucose neuron, etc.). In this study, we further characterize the responses of two chemosensory neurons of the medial styloconica, the RA neuron [canna-sensitive, deterrent neuron identified by Peterson et al. (1993)] and the inositol neuron.
The Inositol neuron was chosen because of its ubiquity among caterpillars and its vigorous response. As a mild feeding stimulant, inositol was chosen to understand its behavioral “meaning” when paired with an effective deterrent. There also have been no medial deterrent studies done on Manduca, where paired deterrent and stimulant were used for linking to behavior. The behavioral “meaning” of inositol may well change with additional complexity of the stimulus mixture, which this study presents the first quantitative measure with other more complex mixtures to follow.

Much of our current information about the sensory and behavioral responses of caterpillars to plant chemicals is based on responses to single compounds. To understand how these chemicals work alone and in combinations, models have been proposed in which spike frequencies from chemosensory neurons responding to phagostimulants (positive behavioral input) are algebraically summed with those responding to phagodeterrents (negative behavioral input) by the decision center in the brain Ma (1972); Blom (1978); Schoonhoven (1987); Hanson et al. (1996); Schoonhoven et al. (1998). The initial central summation hypothesis was based on the assumption that chemosensory neurons are independent parallel channels of information providing faithful representations of the concentrations of individual components. Indeed, independence of two taste neurons mediating opposing stimuli, inositol and caffeine, has been shown in the lateral styloconica of *M. sexta* Glendinning et al. (2000). Our preliminary observations on the medial styloconica of this species, however, suggested that opposing stimuli elicit interacting responses.

Previous studies on sensory responses to plant extracts in several species of caterpillars, including *Manduca*, indicate that such interactions are a common occurrence Dethier and Kuch (1971); Dethier and Cjrmar (1982). Interactions among stimulant and
deterrent taste cells have been documented for several Lepidopteran species Ma (1972); Ma (1977); Blom (1978); Simmonds and Blaney (1984). For example, inositol and sucrose inhibit a sinigren sensitive deterrent cell in both Trichoplusia ni and Mamestra configurata. Additionally, in these two species the inositol cell is suppressed by sucrose but not the reverse, while the KCl sensitive cell is suppressed by inositol and sucrose in M. configurata, but not in T. ni Shields and Mitchell (1995). In the two closely related species of Helicoverpa zea and Heliothis subflexa, the sinigrin sensitive lateral deterrent cell inhibits both the lateral inositol and sucrose cells, but to a differing degree Bernays and Chapman (2000). In these two Heliothine species there is no medial deterrent cell responsive to sinigrin, yet sinigrin suppresses the medial inositol cell, indicating the causation is chemical, not neural. Thus, peripheral interactions among chemosensory cells in caterpillars appear to be a widespread phenomenon Schoonhoven et al (1998).

Not all responses to mixtures result in interactions, however, independence of chemosensory channels has been documented as well. There are examples of different chemosensory channels of information that are not affected by each other’s stimulus. Such is the case in the lateral styloconicum of Manduca, the inositol and deterrent neurons respond independently to mixtures of inositol and caffeine Glendinning et al. (2001). Similarly, our results show that the inositol neuron in this styloconicum is not at all affected by RA in the stimulus mixture. Such differential effectiveness on chemosensory neurons of the same or similar modality appears to be unusual in the literature; for example, a synthetic inhibitor of glucose neurons produces a long lasting inhibition of both medial and lateral glucose cells in Manduca Frazier and Lam (1990), while ascorbic acid produces a shorter inhibition of both inositol and glucose cells Bernays et al. (1998).
In the current study we address the following questions: (1) Are the sensory channels independent for the chemosensory neurons of the medial styloconica as they are for the lateral styloconica? (2) If not, what are the stimulus – response relationships of these neurons to mixtures of opposing stimuli? (3) What is the stimulus – response relationship for the associated behavior?

Methods and Materials

Animals

Eggs of *M. sexta* were obtained from Carolina Biological (Burlington, NC) and reared on the standard wheat germ diet Yamamoto (1969) at 25° C and L:D 16:8. Fourth instar terminal premolts were removed from the culture daily and isolated at room temperature without food and water until used in experiments. For electrophysiological tests, animals were used the day of the molt, whereas those destined for behavioral assays remained in isolation for ca. 24 h and allowed to feed on water-saturated glass fiber filter paper discs for 1 h before the start of the experiment.

Behavioral assays

Feeding behavior was assayed using the two-choice disk test Jermy et al. (1968) modified as in Stitt et al. (1998a) and Rowley et al. (2003). Briefly, behavioral test arenas were prepared with edible Whatman GFA glass fiber filter paper discs of 1.6 cm diameter pinned through pedestals 1 cm above the surface of a black aluminum tray. These discs were wetted with 75 microliters of either a test compound or water control alternating around the inside perimeter of the arena. A video image of each arena was captured at programmed intervals (0.25 h increasing to 1 h); the estimation of food consumption was done by image analysis software in MATLAB™. This automated procedure resulted in greater throughput, improved accuracy, and less observer bias than manual methods.
Twenty-four animals per stimulus were tested in two groups of 12, at least a week apart to minimize brood effects.

The statistic used to quantify feeding behavior was the feeding index (FI), which is a function of the average amounts of the consumption of experimental compound, A, and the water control, B:

\[
FI = \frac{(A-B)}{(A+B)} \quad \text{Eq. 1}
\]

The value of FI falls in the interval from -1 to 1, so a positive or negative number indicates the degree to which the experimental compound stimulates or deters feeding behavior, respectively. An FI was calculated and stored for each animal at each sampling interval.

Because feeding rates vary among animals and across stimuli, data for the FI calculation are acquired when an animal’s consumption reaches a criterion level. In the classical disc test protocol, this occurred when 50% of either the experimental or control discs were consumed, a point in time termed “T\text{50}” Jermy et al. (1968). Because the automated monitoring procedure stores the data, however, any criterion from T\text{10} to T\text{90} can be assigned and calculated post-hoc. Comparisons of the data showed that FI was independent of the time of data acquisition from T\text{33} to T\text{66}; variability within treatment levels was lowest at T\text{66}, however, and therefore it was selected for this study. The point of minimum within-group variability (T\text{66} in this case) provides the most consistent behavior measure when comparing the behavioral differences among binary mixtures. The average time to reach T\text{66} was shorter for stimulants than for deterrents, e.g. 176 min. for 30 mM inositol and 346 min. for 30 mM inositol plus 1.0 mM RA. A few of the animals did not reach T\text{66} before the test terminated (8 h), so the number of animals reaching T\text{66} was not the same for all treatments.
Statistical analysis of feeding data used a one-way ANOVA to determine the significance of differences of average FIs within each mixture concentration level Sokal and Rohlf (1996). Least significant difference (LSD) follow-up tests were conducted when main effects or the interactions were significant. Both correlation and regression analyses were performed to determine which pairs were different and if there was a linear dose-response effect.

**Stimuli**

The chemical stimuli were the same for behavioral and electrophysiological experiments: Rosmarinic acid (ICN) at five concentrations (0, 0.03, 0.1, 0.3, and 1.0 mM) that spanned the behaviorally effective range Simmonds et al. (2006); inositol (myo-inositol, Sigma) at five concentrations (0, 1, 3, 10, and 30 mM) that covered the range of the inositol neuron's dose-response curve Frazier (1986); and KCl (Sigma) at 100 mM. Binary stimuli were the 16 possible combinations of the above RA and inositol concentrations. All experimental solutions contained 100 mM KCl.

**Electrophysiological methods**

The chemosensory responses to stimuli were measured using the standard tip recording technique Frazier and Hanson (1986) with an intact animal preparation Gothilf and Hanson (1994). Briefly, a micropipette with a tip diameter of ca. 50 microns was placed over the pore at the tip of a maxillary styloconicum. The micropipette contained the stimulus made up in 100 mM KCl as the electrolyte in contact with an Ag-AgCl electrode. A high impedance preamplifier (George Johnson, Baltimore MD) possessing a baseline-restoring circuit was used to reduce contact artifacts while preserving spike shape. The preamplifier frequency bandwidth setting was 100 Hz to 3 kHz. An intact larva was anesthetized by immersion in water, and then sealed into a vial with only its head protruding. The vial contained a 100 mM KCl bath making electrical contact between the
body of the larva and an Ag-AgCl reference electrode. In addition to completing the
electrical circuit, the bath served to keep the animal anesthetized for up to eight hours of
recording. A single trial consisted of a one-second recording of the response followed by
0.2 s of voltage calibration pulses. Inter-trial intervals of three minutes permitted full
recovery of the sensory neurons.

Classification of Action Potentials

Individual spikes were extracted from spike trains and classified in a manner that
most accurately associates each AP with the neuron from which it is generated. Each
neuron’s average spike was shown to have a distinct size and shape, a requisite for
discrimination among spikes. Spike variability and noise, however, add uncertainty to the
classification problem and pose challenges for the spike classifier. Therefore we employed
a Bayes classifier, which is theoretically the optimal method for this type of data, and which
in our tests outperformed the classical methods Stitt et al. (1998b); (see also Wheeler (1999)
for an overview of detection and classification methods).

Experimental design and analysis

Each preparation was first tested for functional chemosensory neurons by applying a
standard set of stimuli: 100 mM KCl, 0.03 mM RA, and 1.0 mM inositol. If any of these
failed to evoke an appropriate response, that pair of styloconica was not used. Data from
these standard treatments were analyzed to measure the across-animal variability; no
significant differences among animals were detected.

Animals that responded to the standard treatments were deemed competent and
subjected to one subset of binary mixtures. Each subset of binary mixtures consisted of
the five concentrations of one stimulus and the second stimulus fixed at one
concentration. The 25 treatments with 15 trials each resulted in 375 electrophysiological
recordings from each of the four styloconica of one animal. Each trial was repeated 15 times, allowing 3 minutes between repetitions, for each mixture to obtain a good estimate of within-animal variability. Treatments were presented in order of increasing concentration. Complete data sets from ten competent animals were obtained, one for each of the five RA-inositol sets, and one for each of the five inositol-RA sets.

The phasic and tonic portions of the responses were analyzed separately. The one-second recordings were subdivided into two regions: phasic (first 150 msec) and tonic (remaining 850 msec). From these, a 100-msec window of the phasic region and a 500-msec window of the tonic region were selected for analysis of neural activity. These provided uniform time windows for across-trial comparisons while allowing for the deletion of regions of contact artifact, spurious noise, or momentary loss of electrode contact.

Statistical analysis was used to detect interactions in the neuronal responses to mixtures Sokal and Rohlf (1996). The null hypothesis ($H_0$) tested is that there are no interactions between gustatory cells, i.e., the response of a neuron to a fixed level of its reference compound is not affected by varying the levels of a second compound. To test this $H_0$ for the RA neuron being affected by inositol, a one-way ANOVA compared the average spike frequencies of the RA neuron across the five inositol levels while holding the RA level fixed. This was repeated for each of the five RA sets. This ANOVA determined whether there is a statistically significant ($p < 0.05$) difference between the effects of the two compounds and whether there are any statistical interactions between their effects. The LSD follow-up test for paired comparisons of individual means was conducted to determine if the effects of either compound varied significantly as a function of the level of the other compound. For these LSD analyses, only within-level mean comparisons were made. We
also tested the $H_0$ for the inositol neuron being affected by RA by reversing the roles of inositol and RA described above.

**Results**

*Chemosensory responses of RA-sensitive neuron to RA*

A sensory neuron in the medial styloconicum is strongly activated by low concentrations of RA (Fig. 1A.b). After onset of the stimulus, the spike frequency increases slowly after a delay of approximately 10 msecs (Fig. 2A), a pattern that is characteristic of deterrent neurons of many lepidoptera Schoonhoven and vanLoon (2002). The RA sensitive neuron appears to have a threshold of activation below the 0.3mM level, the lowest level tested. The positions of the first points in each of the four panels plotted in Fig. 3A (Fig. 3B) map the phasic (tonic) dose-response curves and show the dose-dependency of the RA neuron over the concentration range from 0.03 mM RA to the 0.3 mM level.

Insert Figures 1 and 2.

In the phasic region of the response, this neuron saturates at about 70 spikes/sec and about 60 spikes/sec in the tonic region. This cell changes its spike shape during the phasic portion of response, but the Bayesian classifier handles these changes with less than two percent error Stitt et al. (1999). The overall response to the concentration series is best seen in the 3D surface plots of Figures 3A and 3C. A cell in the lateral styloconicum showed some response to RA (Fig 1A.b), but no dose response effect, so we do not consider it a RA sensitive neuron.
Chemosensory responses of inositol-sensitive neurons to inositol

A second sensory neuron in the medial styloconicum responds vigorously to inositol (Fig. 1A.c). During the phasic region of this response, the spike frequency increased quickly to a peak well within the first 50 msec (Fig. 2B), a pattern that is typical of the classic inositol neuron in many lepidopteron species Bernays and Chapman (2000); Glendinning et al. (2001); Schoonhoven and van Loon (2002). The response is dose-dependent and saturates at about 200 spikes/sec at 30 mM in the phasic region (Fig. 4B). In the tonic region, the spike frequency of this neuron is relatively stable and decrements slowly over time (Fig. 2B). The response is dose-dependent and saturates around 125 spikes/sec at or above 3 mM (Fig. 4D, circled points).

Chemosensory responses to stimulus mixtures

The RA neuron is slightly affected by the inositol in stimulus mixtures, but only in the phasic region at the highest two concentrations of RA (last 2 panels of Fig 3B). In contrast, the inositol neuron is greatly affected by RA in stimulus mixtures, the spike frequency was reduced by 50% or more in both the phasic and tonic regions by the addition of RA ≥ 0.03 mM (Fig. 4). In most cases, maximal inhibition is seen even with the lowest concentration of RA tested, with little or no further increase in inhibition with higher RA concentrations. This is best illustrated by the 3D surface plots of Figs. 4A and C which show a steep drop to a lower response level when RA is present in the stimulus mixture.

Thus, the above data show the inositol neuron is strongly inhibited by RA in the medial styloconica. In contrast, in the lateral styloconicum which has no RA sensitive neuron, the inositol neuron showed no evidence of interactions. Responses to mixtures of
inositol and RA were not significantly different from those to inositol alone (Fig. 1B.d vs. 1B.c).

**Insert Figure 5**

*Spike-epoch correlation*

In addition to documenting interactions resulting in changes in spike frequency, we also searched for temporal interactions such as phase locking of spikes from the two neurons. No evidence of this was found in any of the 240 recorded trials of responses to mixtures. A joint interval analysis of spikes from these RA-inositol mixtures combined with a single factor ANOVA yielded no difference between the forward and backward histograms (analysis not shown). A representative spike train segment is shown in Fig. 5 in which the inositol neuron is firing slightly faster than the RA neuron and "walks through" a complete cycle of the latter with no discernible temporal interactions, thus clearly demonstrating the absence of peripheral phase-locking between these two taste neurons.

*Behavior*

Feeding behavior data from 500 larvae tested on mixtures of RA and inositol are shown in Figs. 6 and 7. Figure 6 illustrates fixed levels of RA and varying levels of inositol, while the reverse is shown in Figure 7. Both figures show the single compound dose response curves as the first point in each panel.

**Insert Figures 6 and 7.**
RA is clearly a strong phagodeterrent, with a threshold for deterrency between 0.03 and 0.1 mM, and a dynamic range extending to 1 mM (Fig. 7, panel 1). These dose-response curves become less steep with increasing inositol concentrations in the mixtures (Fig. 7, panels 2-5). While inositol by itself is a weak phagostimulant (Fig. 6, panel 1), in mixtures it is more effective at counteracting deterrency than expected. Inositol-RA mixtures are much less deterrent than RA alone (note steeper dose-response curves in mixtures with RA 0.1 and 0.3 mM, Fig. 6).

Because all experimental stimuli contained 100 mM KCl to maintain comparability to the electrophysiological study, we looked for effects of this level of salt on behavioral responses. Results show that 100 mM KCl is slightly but significantly deterrent compared to the water control. For example, in the first plot in each of Figs. 6 and 7, the feeding index at 0 inositol and 0 RA (which contain 100 mM KCl) is slightly negative. Interestingly, low concentrations of RA counteract this inhibition (Fig. 7, first three panels: second value is higher than the first).

**Discussion**

With the goal of understanding the quantitative relationships of styloconic chemosensory inputs regulating feeding behavior in *Manduca sexta*, we have obtained associated behavioral measures and electrophysiological recordings from peripheral taste receptors responding to inositol, RA and their mixtures over their effective concentration ranges. By correlating sensory and behavioral responses to mixtures we determined that interactions occur at the peripheral level and we conclude that central level interactions are also present and contribute to the behavioral decision-making process.
Sensory Neuron Responses

Responses to canna extract and to RA both have a relatively long latency, a slowly increasing spike frequency during the phasic region and a relatively low-frequency tonic response similar to the medial styloconica response to aristolochic acid Glendinning et al. (1999). These characteristics have also been seen in sensory neurons referred to as labeled line deterrent neurons in other caterpillar species Ma (1972); Blom (1978); Schoonhoven and van Loon (2002). The *Manduca* medial deterrent cell is very sensitive to RA, but its threshold is not as low as the exemplary "specialist" deterrent cell seen in more monophagous species Schoonhoven and van Loon (2002).

The inositol and KCl neurons’ responses are consonant with those obtained by many workers Frazier (1986); Peterson et al. (1993); Glendinning et al. (2000); Schoonhoven et al. (2002) but somewhat different than that of del Campo and Miles (2003), who show a much higher frequency response by the KCl neuron.

Peripheral Interactions

The presence of RA in the stimulus mixture significantly decreases the medial inositol neuron's spike frequency (see Fig. 4.A and C). Both the phasic and tonic regions of the response show comparable relative decrements, indicating that the inhibition is present from the onset of the stimulus. Whether this is a chemical effect of RA blocking the inositol cell or an electrical effect of the deterrent cell altering the firing threshold of the inositol cell is not known; studies of other caterpillar species suggest these effects are chemical interactions at the dendrite level Schoonhoven et al. (1998). Thus RA decreases the phagostimulant (inositol cell) input to the CNS while simultaneously encoding phagodeterrent input, thereby presumably producing a dual action leading to increased phagodeterrency.
The reverse of this interaction is also true, although to much less of an extent. The presence of inositol in the stimulus mixture significantly decreases the RA cell response as a function of increasing inositol concentration, but only at higher concentrations of RA. While this effect produces a slight reduction in the RA cell response, it too is present from the onset of the response and is likely to be the result of chemical interactions at the dendrite level. Thus, inositol has dual actions. It encodes phagostimulant input to the CNS while simultaneously decreasing phagodeterrent input, but only at higher levels of phagodeterrency.

One of the objectives of this study was to determine whether sensory inputs are completely independent or whether interactions at the periphery modify individual cellular responses and thus contribute to candidate sensory codes. For example, if the sensory inputs are independent and no interactions are seen, the only data needed to understand the response to complex stimuli would be the dose-response curves of the individual components. This is the case for the inositol and RA relationships in the lateral styloconicum (Fig. 1B.d). If interactions do occur, however, the mixtures themselves must be tested and the interactive effects characterized. Our data show that peripheral interactions between stimulant mixtures do occur, as is the case for inositol-RA mixtures in the medial styloconicum (Figs. 3 and 4).

This inhibition by RA of the inositol neuron of the medial, but not that of the lateral, represents a case of differential secondary effects of a compound on neurons of the same modality. This appears to be unusual in the literature, as most examples of inhibitory compounds produce similar affects on chemosensory neurons of the same or similar modalities. For example, a synthetic inhibitor of glucose neurons produces a long lasting inhibition of both medial and lateral glucose cells in Manduca Frazier and
Lam (1990), while low pH (via ascorbic acid) partially inhibits inositol and glucose neurons in both medial and lateral styloconica Bernays et al. (1998).

**Inhibition of KCl Response**

The presence of RA at increasing concentrations also produces inhibition of the KCl sensitive cell (compare Figs. 1Ab and 1Aa). This is also evident in other studies of deterrent neurons of *Manduca* (see Figs 4 and 5 of Glendinning et al. (1999); Schoonhoven et al. (2002), where increases in responses of the lateral, medial, and epipharyngeal deterrent cells correlate with reduced salt cell firing. Thus, if the salt-sensitive neurons also provide input that is inhibitory to feeding, the deterrent cells’ activity (or the deterrent chemical itself) would have an additional modifying effect on behavior, namely to inhibit this inhibition. Indeed, our data show an increase in feeding index as RA is increased from zero to 0.03 mM (Fig. 7, first three panels).

**Sensory Neuron Phase Locking**

Other types of interactions, such as by spike phase locking, has been seen in chemoreceptor neurons of some insects White et al. (1990); Mitchell and Sutcliffe (1984), but to our knowledge not in caterpillars. Our large data base of sensory information on *M. sexta* provides a unique opportunity to carefully examine this possibility. Rigorous analysis showed, however, that spike phase locking does not occur between the inositol and RA neurons (Fig. 5).

**The Importance of the Phasic - Tonic Distinction**

The phasic (first 150 msec) and tonic (next 850 msec) portions of the one-second recorded sensory responses clearly differ in spike frequency (Figs. 3 & 4), but is this only a physiological distinction or are there behavioral correlates as well? Although behavior and
electrophysiology have not been recorded simultaneously in Manduca, our personal observations of video recordings of initial encounters with leaves indicate that both tapping and dragging of the maxillary styloconica occur, suggesting that both phasic and tonic (respectively) responses are utilized.

In other species such as the blowfly, this question has been resolved by experiments employing simultaneous behavioral and electrophysiological recordings with the requisite time resolution. For example, Dethier (1968); Dethier (1976) showed that in Phormia regina, only 14 spikes in the first 70 msec are sufficient to activate the proboscis retraction muscles in a rejection response to a deterrent (0.5 M NaCl). This response fails if the interspike interval lengthens Getting (1971), indicating that the high spike frequency of the phasic portion is the critical factor in eliciting this behavior. In contrast, discriminations among similar sucrose concentrations require 500 - 1000 msecs in contact with the stimulus, indicating that extensive data from the tonic phase is needed for finer discriminations Smith et al. (1984). Thus in the fly, phasic and tonic portions of the sensory response subserve different functions -- rapid response vs. fine discriminations. In Manduca, Dethier and Cjrnar (1982) observed that five sec or more is required to decide between two host plant saps; obviously, more sensory information is needed for fine discrimination in caterpillars also, but whether this is obtained by many rapid taps (phasic) or long drags (tonic) is not known. Finally we note that other behavioral assays, e.g., frequency of biting, duration of first biting phase, meal duration, etc. show different correlations with sensory input, and the importance of the phasic-tonic distinction may vary with each Ma (1972); Frazier (1986); Glendinning et al. (2001).
Relating Behavioral Outputs to Sensory Inputs

To better understand the relationship between feeding behavior and its relationship to the chemosensory response, the entire data set of 420 behavioral experiments and 375 electrophysiological recordings were statistically analyzed. The 1-second recording from the styloconica provides an estimate of the sampling (biting rate is 1 to 3 per second, unpublished observations). Several studies have correlated styloconica inputs with continued food choice using several different behavioral techniques, for example Schoonhoven and Blom (1988); Frazier (1986); Blom (1978); Ma (1972). There are other inputs, certainly, but this provides a beginning point to quantify the input-output relationships, as we have indicated in a previous conceptual model of this approach Hanson, et al (1996). RA is not toxic to Manduca, and the ingestive behavior is highly reversible and repeatable over successive timeframe measurements. Because of the large data sets and our confidence in assigning resolved spikes to their source neuron, the phasic and tonic portions of the spike trains could be independently correlated with behavior.

Correlation of the feeding index with the phasic portion of the sensory response showed the coefficients of inositol and RA to be roughly equal in magnitude but opposite in sign as expected when comparing the effects of stimulants and deterrents (Table 1). Similar findings were obtained for the tonic portion of the response (Table 1), which is probably a more reliable measure as it is based on a greater number of spikes. The cross product terms (interactions) of this correlation are small, indicating that the main effects on behavior are the neuronal inputs. The largest coefficient is only about 0.5 (Table 1), suggesting that much of the sensory response does not correlate with behavior, and/or factors other than those measured are also involved in the feeding decision.
The overall mathematical relationship between the observed feeding behavior and the sensory responses was determined by regressing the feeding index on the firing rates of both chemoreceptors for each compound alone and for each binary mixture. Fitting a polynomial function to this regression showed that the overall relationship is a linear one, as the quadratic terms were insignificant:

\[ FI = (0.6191 \times \text{inositol spike frequency}) + (-0.2858 \times \text{RA spike frequency}) - 0.3177 \]  Eq. 2

The regression analysis estimated the parameters of the linear dependence of feeding behavior upon electrophysiology, as shown in Eq. 2 \( (R^2 = 0.31; \text{F-Statistic} = 3.12; p < 0.05) \). The value of the constant term (-0.3177) accounts for the mild feeding deterrence of 100mM KCl as well as the overall deterrence of RA.

**Conclusions**

Rosmarinic acid, the deterrent principle of *Canna generalis* and other deterrent plants, is a moderately strong phagodeterrent for *Manduca sexta* caterpillars that activates a chemosensory neuron in the medial maxillary styloconica. No deterrency is seen at low spike frequencies of this neuron, but at 30-35 spikes/sec (40-50% relative activity), a threshold is reached above which feeding deterrency increases linearly with spike frequency. At these activity levels, the lower mean firing rate of the medial styloconic RA neuron is approximately a factor of three lower than the medial inositol sensitive cell (Fig. 3 and 4), yet it had a much larger effect on behavior (Figs. 6 and 7). The magnitude of change in behavior index per unit neural activity for RA is a
factor of six greater than that for inositol suggesting a large central weighting factor for RA input (Fig.6 and 7). Peripherally, the RA neuron (or RA itself) causes substantial inhibition of the inositol neuron; the reverse is true in a limited range of concentrations. Electrical interaction in the form of phase-locking of spikes was not detected. Thus we conclude that the observed peripheral interactions occur at the transduction level, but post-transduction channels of information are independently transmitted to the brain. Furthermore, the presence of such strong interactions indicates that correlations of behavior with total spike activity (as opposed to activity of individual identified neurons) may be highly inaccurate when mixtures are the experimental stimuli.

Although inositol alone is only a mild feeding stimulant, in mixtures with RA its phagostimulation is much greater than would be predicted from the known sensory inputs. Apparently, the simultaneous stimulant and deterrent inputs elicited by mixtures more strongly activate the feeding decision center than would the sum of these inputs obtained singly.

Because feeding behavior correlates equally well with phasic and tonic responses, we conclude that the chemosensory information in the first 150 msec is sufficient for discrimination, at least for these antagonistic stimuli. The tobacco hornworm probably taps and drags its styloconica on the surface and cut edges of leaves being considered for food, each phase of the response may contain input information important to the feeding decision, although we could not demonstrate it here.

The overall relationship between sensory activity and behavior is generally linear as shown by correlation and regression analysis, thus reinforcing the classic paradigm of central summation of positive and negative inputs controlling the level of feeding. A more precise measure of this relationship would include aspects such as the deterrence threshold,
central weighting factors, and phasic or tonic responses. Future work will involve the
development of a model which incorporates these parameters into a descriptive sensory-
behavioral transformation capable of quantifying sensory control of feeding behavior.

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Figure Legends

**Figure 1**: Sensory neuronal responses of the styloconica to KCl, RA and inositol. One-half second segments from the tonic phase (150-650 msec) of spike trains the amplitudes of which were normalized to 1024 samples/mV. All stimuli included 100 mM KCl as the electrolyte for these and all subsequent figures.

**Figure 2**: Time courses of typical responses by the RA and inositol neurons of the medial styloconica. **A.** Response to 0.3 mM RA. **B.** Response to 1.0 mM inositol. Left ordinate: normalized amplitude (action potentials). Right ordinate: normalized instantaneous frequency (asterisks).

**Figure 3**: Effect of inositol on the RA neuron. **A.** Surface plots of phasic portion of spike trains of responses to RA, inositol, and their mixtures. **B.** Individual dose-response curves forming these surface plots. **C.** and **D.** Plots of the tonic phase of spike trains used in **A.** and **B.**

**Figure 4**: Effect of RA on the medial inositol neuron. Details as in Figure 3.

**Figure 5**: Absence of spike-epoch correlation between RA and inositol neurons. Responses to 0.3 mM RA in 10 mM inositol by these two neurons show no phase-locking between spikes as the RA spikes “walk through” the inositol spikes.

**Figure 6**: Feeding behavior to inositol, RA, and their mixtures. Within each panel, the behavioral response to increasing concentrations of inositol is plotted for a fixed level of RA.

**Figure 7**: Feeding behavior to RA, inositol, and their mixtures. Within each panel, the behavioral response to increasing concentrations of RA is plotted for a fixed level of inositol.
Table 1 – The correlation coefficients between behavior and the average spike frequency from the phasic (tonic) portion of the electrophysiology are shown along the second (third) row of the table. The values of correlation between behavior and reference compound EP were in general significant (i.e., p <= 0.05). There was no correlation between behavior and the EP interaction terms in either phase.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Inositol EP</th>
<th>RA EP</th>
<th>Inos*RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phasic</td>
<td>0.5061 (p &lt; 0.01)</td>
<td>-0.4956 (p &lt; 0.05)</td>
<td>0.1415</td>
</tr>
<tr>
<td>Tonic</td>
<td>0.4095 (p &lt;0.05)</td>
<td>-0.3357 (p = 0.10)</td>
<td>0.0044</td>
</tr>
</tbody>
</table>