Chemoreception of Sucrose and Amino Acids in Second and Fourth Instars of the Spruce Budworm, *Choristoneura fumiferana* (Clem.)

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ABSTRACT

Chemoreception of Sucrose and Amino Acids in Second and Fourth Instars of the Spruce Budworm, *Choristoneura fumiferana* (Clem.)

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Electrophysiological responses from various chemosensilla on the maxillae of second instar spruce budworm larvae were obtained using sucrose and l-amino acids as stimuli. Complementary tests stimulating the L1 on the maxillary palp and the lateral styloconic sensilla (LST) on the galea of fourth instars were also performed. The results from both second and fourth instars were then compared with earlier electrophysiological work done on older sixth instars. Sensitivity of responses from the sugar-sensitive cell of the L1 and the LST to sucrose for second, fourth and sixth instars revealed that the mean firing frequency of this cell remains unchanged in the L1 sensillum during larval development. However, for the LST an age-related sensitivity to sucrose was noted with increases in larval age.

The LST and the L2 sensilla of second instars were stimulated with thirteen amino acids; however, only three (l-leucine, l-aspartic acid and l-valine) evoked a response in the former sensillum. No responses were obtained from the L2 sensillum to any of the amino acids, confirming that this sensillum does not possess an amino-acid sensitive cell, but instead has a water-sensitive cell, which is inhibited by higher concentrations of KCl. Further analysis of responses from the l-proline-sensitive cell of the medial styloconic sensillum (MST) revealed that the second instar larvae are less

sensitive to stimulations with l-proline than are fourth and sixth instars. These results are discussed in relation to the changes in feeding preferences of larvae as they age.

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I dedicate this thesis to my future baby, who has already set up my new goal in life.

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Introduction:

Over the past few decades, there has been increasing interest in the study of insect chemoreception and the possible mechanisms of coding of impulses from taste sensilla. While much of the early work on insect gustation was done on flies (Dethier and Hanson, 1965; Shimada et al., 1974; Wieczorek and Köppl, 1982; Cheung and Smith, 1998) there has been increasing interest in phytophagous caterpillars; these are well equipped with chemosensilla that they use to assess the chemical composition of the host plants prior to ingesting them (Schoonhoven and Van Loon, 2002). The plant's chemical composition is the most important source of information used by phytophagous insects when assessing potential food sources (Schoonhoven and Van Loon, 2002; Guertin and Albert, 1993) and it is through the use of the insect's chemosensilla that chemical information gets translated into signals that lead to behavioural responses (Simmonds and Blaney, 1990). Thus, the feeding behaviour of insects, as well as of many other animals, is influenced by the neural input received from their chemosensilla (Blaney et al, 1986). Although factors such as light, temperature, humidity and leaf physical traits are important features that can affect host plant selection, food plant acceptance and maintenance of feeding is ultimately influenced by the chemical features of the plant (Heron, 1965).

Chapman (2003) summarized our current knowledge of insect contact chemoreception in caterpillars. He primarily focused on the idea that food acceptance and rejection in caterpillars depends on the balance of positive (stimulant compounds) versus negative (deterrent compounds) inputs, which are detected by gustatory receptors and

assessed by the central nervous system. He points out that upon contact with a single chemical to which a neuron is sensitive, specific information is transmitted via electrical impulses into the central nervous system. This is considered as the basic labeled line system of an insect's taste neuron. More complex signals are received by the CNS when various neurons simultaneously make contact with mixtures of compounds whose signals get transmitted by the axons of each of those neurons. These signals are then conveyed to the CNS, and interpreted as an across-fiber pattern. Chapman (2003) also points out that whatever the effects a chemical has, positive or negative, on a neuron's response, this response can be greatly affected by the presence of other chemicals and this in turn will influence the feeding behaviour of the insect. Although much of the physiological data from chemosensilla can be directly correlated to observed behavioural responses (Ishikawa, 1967; Albert and Parisella, 1988*b*; Glendenning et al., 2000), Chapman (2003) emphasizes the feeding behaviour of caterpillars cannot always be explained by the information from gustatory chemoreceptors. He furthermore stresses that a great deal of work is still required in order to make clearer sense of the precise role of the gustatory system in mediating the feeding decision process, especially since great variability in terms of feeding preferences are observed at different stages of larval development.

The spruce budworm larva, *Choristoneura fumiferana* Clem. (Lepidoptera: Tortricidae), is one of the few lepidopterous insects that have been studied with a view to gaining a good understanding of the mechanisms by which feeding decisions are made. Since it is still a potential pest for the evergreen forests of eastern Canada and the U.S.,

there is continuing interest in expanding our knowledge of the chemosensory mechanisms of host plant selection in this insect species.

The spruce budworm (SBW) is one of the most important forest defoliators in North America, and has caused significant tree mortality over millions of hectares of softwoods (Miller and Rusnock, 1993). In eastern Canada, the preferred host species are balsam fir ((*Abies balsamea* (L.) Mill.) followed by white spruce (*Picea glauca* (Moench) Voss.), red spruce (*Picea rubens* Sarg.), and black spruce (*Picea mariana* Mill.) (Lawrence et al., 1997). This order of preference is predominantly determined by phenological characteristics of the hosts. Balsam fir and white spruce bud breaks coincide with the time when second instar larvae end their diapause in Spring (Albert and Jerret, 1980; Mattson et al., 1991), while in black spruce it occurs much later so that these latter hosts generally experience a lower mortality (Mattson et al., 1991).

The SBW is univoltine, undergoing one generation per year. Development begins with the first instar that has a short duration of approximately 3 days (McGugan, 1954). This stage, described by Han et al., (2000), is divided into four phases: the egg hatching, construction of hibernaculum, excretion of frass pellets and moulting into the second instar. Evidence suggests that no feeding occurs throughout the first larval instar (Morris, 1963) although a recent study by Retnakaran et al., (1999), reported that first instar larvae do engage in slight nibbling when placed on the surface of balsam fir needles. Most feeding activity in the SBW, however, is known to occur from the second through the

sixth instars, with the last three instars causing the most damage on the host trees (Morris, 1963; Miller, 1975).

The SBW overwinter as second instar larvae that eventually emerge from diapause with the onset of warmer temperatures during the month of May (Régnière, 1990). Second instars mine previous years' needles and either feed on staminate cones or enter the buds; soon after bud opening, the larvae emerge and continue to feed from needles as third instars. Larval development proceeds until the end of the sixth instar when feeding stops in preparation for pupation (Retnakaran et al., 1999). Caterpillars pupate and emerge as adult moths 8 to 12 days later, initiating mating about 24 hours after emergence. Females select suitable oviposition sites, normally on the undersurface of one-year old needles, and lay their egg masses, and eggs hatch in 8 days (Outram, 1971).

In lepidopterous caterpillars, host plant selection behaviour is primarily controlled by the activity of taste sensilla located on the mouthparts (Schoonhoven and Van Loon, 2002; Blom, 1978). The epipharynx on the inner surface of the labrum contains two dome-shaped sensilla, each innervated by three chemoreceptor neurons (Schoonhoven and Van Loon, 2002). The galea on each maxilla bears two elongated uniporous pegs. They are commonly referred to as the lateral (LST) and medial (MST) styloconic sensilla and each is innervated by four chemoreceptor neurons, a number that remains constant among all studied caterpillars (e.g. tobacco hornworm *Manduca sexta* (De Boer et al., 1977), the silkworm *Bombyx mori* (Ishkawa, 1967), the white cabbage butterfly *Pieris*

brassicae (Schoonhoven and Van Loon, 2002), the spruce budworm *Choristoneura fumiferana* (Panzuto and Albert, 1997)). Removal of styloconic sensilla from both tobacco hornworm *Manduca sexta* and silkworm *Bombyx mori* larvae results in the impairment of the insect's food detection ability, making the role of these sense organs very important during the selection of suitable feeding sites (De Boer et al., 1977; Ishkawa, 1967). The maxillary palpi bear a group of eight smaller sensilla involved in olfaction or gustation (Schoonhoven and Van Loon, 2002). The responses of individual chemosensilla within this group have only been studied in sixth instar SBW larvae (Albert, 2003; Hock, 2005). Three of these sensilla are known to be gustatory in SBW larvae, and they are referred to as L1, L2 and A3 (Albert, 2003). Although the exact number of chemoreceptor neurons innervating each sensillum has not yet been determined, a group of at least 20 nerve cells are found at the proximal segment of the palp (Albert, 1980).

Each chemoreceptor neuron can respond to a variety of compounds, a feature known as the sensitivity spectrum of its receptor cells (Schoonhoven and Van Loon, 2002). A common approach in the study of chemoreception is to identify receptor neurons according to the substance(s) (chemical compounds) that elicits the highest degree of stimulation (highest number of impulses/s) (Schoonhoven and Van Loon, 2002). For instance, the LST of SBW larvae contains a water-, a salt-, an amino acid- (except l-proline) and a sugar-sensitive cell (Albert and Parisella, 1988*b*; Panzuto and Albert, 1997, 1998). In the MST, one cell responds to water, two cells respond to salts and one responds only to l-proline (Panzuto and Albert, 1998). Albert (2003) has

identified the presence of at least two cells in the L1 sensillum, one responding to water and the other to sugars. No characterization has yet been done on the A3 sensilla and, only recently, the presence of a water-sensitive cell was confirmed in the L2 sensilla of sixth instars (Albert, unpublished).

No other work apart from Panzuto and Albert (1997) and Hock (2005) has focused on the receptor binding characteristics of receptor cells in lepidopterous caterpillars. Primarily based on the work of Shimada (1974) on fleshflies, Panzuto and Albert (1997) found that the sugar cell of the LST of SBW larvae responds to stimulation by pyranose (e.g. glucose) and furanose (e.g. fructose) sugars, which are known to bind to P- and F-sites respectively (Panzuto and Albert, 1997). Furanose sugars also elicit responses from the sugar-sensitive cell of the L1 sensillum of sixth instars. However, no responses from this sensillum were obtained when stimulated with pyranose sugars, thus demonstrating the absence of P-sites on the sugar-sensitive cell of this sensillum (Hock, 2005).

From electrophysiological tests, responses from individual neurons in a chemoreceptor can be obtained by visually discriminating the shape of the action potential (spike shape). For instance, recordings from SBW larvae from stimulations with sugars, amino acids and water induce action potentials that are monophasic positive with differing amplitudes, while those from stimulations with salts are biphasic with both positive and negative flanks (Schnuch and Hansen, 1990; Simmonds and Blaney, 1990).

Studies of the responses from various gustatory chemosensilla in older SBW larvae have revealed a good deal of information regarding their possible functions. An electrophysiological study of the LST showed a strong correlation between behavioural feeding responses of sixth instar larvae and sucrose responses from the sugar-sensitive cell of that sensillum (Albert, 1982; Albert and Parisella, 1988*b*). The behavioural threshold for sucrose was found between 0.1 and 1.0 mM l^{-1}. At the physiological level, the neuron's threshold was < 0.5 mM l^{-1} and the cell reached a plateau of firing frequency at the 50 mM l^{-1} concentration. The concentration of sugar levels found in the host plant is approximately 37 mM l^{-1} (Little, 1970), which fall within the range of detection of the sugar-cell neuron in the LST. This corroborates the importance of the LST in mediating feeding behaviour in SBW larvae.

The sugar-sensitive cell in the L1 sensillum of sixth instars was shown to have a higher electrophysiological threshold (1 and 10 mM l^{-1}) and a higher plateau (200 mM l^{-1}) than the one in the LST. It may thus function as a detector of higher sugar concentrations normally found in plants that are stressed (Albert, 2003). A similar function has been ascribed to the l-proline sensitive cell in the MST (Panzuto and Albert, 1998). Concentrations of carbohydrates and amino acids, including l-proline, are known to increase during periods of plant stress (Zwiazek and Blake, 1990) and in black spruce these compounds are known to be involved in osmoregulation. In Scots pine (*Pinus sylvestris* L.) the increase in proline concentrations during Spring and early Summer has been associated with drought stress (Näsholm and Ericsson, 1990). Evidence suggests

that stressful conditions in plants increase their susceptibility to herbivorous insects, resulting in higher chances of insect outbreaks (White, 1984; Louda et al., 1987).

Sugars, important components for energy production and other metabolic functions, and amino acids, critical elements for the growth of all organisms, are known in many cases to act as strong phagostimulants (Schoonhoven and Van Loon, 2002). Heron (1965) was the first to evaluate the chemotactic responses of the SBW larvae, and he concluded that a number of sugars as well as the amino acid l-proline were the most effective feeding stimulants for the larvae. Feeding preference studies using host plant extracts showed that sugars/glycosides were the most stimulating fractions followed by the amino acids/bases fractions; however, combining the two host-plant fractions resulted in an even greater response than each of the individual fractions alone (Albert and Parisella, 1985). Evaluating the responses of sixth instar SBW larvae revealed that the most preferred sugars are sucrose, fructose, m-inositol, and glucose (Albert et al., 1982) and the most preferred amino acids are l-proline, l-serine, l-alanine, and l-lysine (Albert and Parisella, 1988).

Harvey (1974) studied the effects of sugars on SBW larval development and survival. He demonstrated that larvae reared on sugarless diets had longer developmental times and lower survival rates compared to those reared on diets containing readily utilizable sucrose, fructose, glucose and raffinose (Harvey, 1974), the principal sugars of developing balsam fir (Little, 1970) and white spruce foliage (Chalupa and Fraser, 1968). Furthermore, Clancy et al. (1988) suggested that one of the key factors affecting

budworm performance is the acquisition of a balance between N and mineral elements. The importance of nitrogen consumption by herbivorous insects has also been reviewed by Mattson (1980).

The nutritional requirements of insects change over time by virtue of varying demands such as growth, reproduction and migration. Since caterpillars' growth requirements decline over time while energy storage needs increase, a shift from nitrogen to carbohydrate demands is expected over successive instars (Stockhoff, 1993). In SBW larvae this change in nutrient requirements seems to parallel changes in foliar nutrient levels occurring during the growing season of its balsam fir host trees (Albert and Bauce, 1994). For instance, total sugar concentrations increase as needle expansion progresses (Little, 1970) and decrease in 1-yr-old needles in balsam fir and white spruce (Guertin and Albert, 1994). Moreover, a substantial decline in levels of N during the growth of new needles is a common feature of woody plants (Clancy et al., 1988).

In SBW larvae, changes in nutrient demand in relation to larval age were first studied by Harvey (1974). He evaluated the effects of high versus low sugar and nitrogen diets on larval development and found that diets containing higher levels of sugars were more beneficial for older larvae than for younger ones, whereas the opposite was true for nitrogen containing diets. Likewise, an age-related nutritional change was noted when sixth and fourth instars were given a choice of extracts from lateral and terminal shoots of balsam fir. Analysis of sugar and nitrogen contents from lateral versus terminal shoots revealed that sugars are found at higher levels in lateral shoots and nitrogen levels are

higher in terminal shoots. Sixth instars preferred extracts from lateral shoots of young (30 yr old) balsam fir trees while fourth instars preferred extracts from terminal shoots of both young and old (70 yr) trees (Albert and Bauce, 1994). Similar changes in dietary selection have been observed in the gypsy moth larvae, in which nitrogen to lipid preferences are shown to shift during larval ontogeny (Stockhoff, 1993). Larval age is an important factor influencing the feeding behaviour of SBW caterpillars (Guertin and Albert, 1994; Panzuto and Albert, 1998).

The feeding behaviour of insects should assure a balance of nutrient intake; this is achieved in part by the recognition of specific nutrients in the food source by the gustatory system. Thus, changes occurring at the receptor level can drastically modulate the feeding decisions of the animal; moreover, internal feedback mechanisms such as regulation mediated by the action of hormones or nutrient content within the haemolymph (insect's blood) provides a constant update of the insects nutritional status (Simpson and Raubenheimer, 1993).

Among the factors that are known to modify receptor sensitivity, which in turn can influence the insect's feeding behaviour, are time of day, nutritional status, previous experience and larval age (within and between instars) (Schoonhoven and Van Loon, 2002; Blaney et al., 1986). Normally, chemoreceptor variability can be monitored by changes in the firing frequency of receptor cells to given concentrations of a solution (Blaney et al., 1986). A comparative electrophysiological study between older and younger SBW larvae shows that responses from the LST of sixth instars by sucrose,

fructose and m-inositol are significantly higher than those of fourth instars (Panzuto and Albert, 1997). In addition, these results are strongly correlated with previous behavioural observations (Albert et al., 1982). Similarly, electrophysiological responses to amino acids are higher for the older larvae than for the younger ones (Panzuto and Albert, 1998); however, no correlation is found with the behavioural preferences previously obtained on sixth instars by Albert and Parisella (1988).

Up to now, all electrophysiological research conducted on caterpillars, including the SBW larvae has concentrated on the sensory responses from older instars, specifically fourth and sixth instars in the case of the SBW (Albert and Parisella, 1988*b*; Panzuto and Albert, 1997; 1998; Albert, 2003; Hock, 2005). This is likely because most significant host-plant damage occurs during these older larval stages. In addition, it is easier to work with these instars due to their larger size (e.g. sixth instar SBW larvae = approx. 12 mm long; second instar SBW larvae = approx. 1mm long).

Having a more complete set of information on the response characteristics of chemoreceptor neurons of each sensillum for every larval instar could be important as no data of this sort has ever been documented in any kind of insect. By acquiring a greater understanding of the sensory system of younger caterpillars, we could gain a better insight of the coding mechanisms involved in food selection behaviour. This ultimately could lead to the development of more efficient pest control programs targeted directly to younger larval instars. The application of insecticides has normally focused on eliminating SBW only after the larvae have started feeding vigorously on the needles (3rd

to 6th instars) (Retnakaran et al., 1999). However, being able to apply behaviour modifying compounds such as feeding deterrents at early stages of larval development, could provide an effective way to reduce damage to trees (Retnakaran et at., 1999).

The primary objective of my thesis is to obtain electrophysiological recordings from second instar larvae using different compounds known to stimulate older instar larvae to determine if their physiological responses vary from those of fourth and sixth instars. Electrophysiological responses will be obtained from the sugar-sensitive cells of the LST and the L1 sensilla of second and fourth instars, primarily to stimulation with various sucrose concentrations. Additionally, responses to l-amino acids will be obtained from the LST, MST and L2 sensilla on second instar larvae. Finally, comparisons between and within instars will be conducted for responses of each sensillum using data from the present and previous work conducted on older larvae (Albert and Parisella, 1998*b*; Panzuto and Albert, 1997; 1998; Albert, 2003). Since the nutritional requirements of the larvae are assumed to shift from nitrogen to carbohydrates as they grow, I expect to observe lower electrophysiological responses to sucrose than to amino acids on second instar larvae compared to the older ones. This will allow us to gain a better knowledge of the physiological response characteristics of chemoreceptor sensilla of second, fourth and sixth instar larvae.

Materials and Methods

Insects

Insects were obtained as post-diapausing second instar larvae from the Forest Pest Management Institute, in Sault Ste- Marie, Ontario. Upon arrival to the lab, some larvae were placed in a Petri dish inside a refrigerator at a temperature of 4° C to slow down their developmental rate. The remainder were reared on an artificial diet following the procedures used by Grisdale and Wilson (1988) until they reached the fourth instar. Larvae were kept in an incubator at 25° C and a relative humidity of 70% with a photoperiod of 16L:8D.

Second instars were used in all experiments except in experiment 3 where fourth instars were used.

Electrophysiology

All insects were prepared following the methods used by Albert (2003). Larvae were sectioned behind the third thoracic segment and the anterior end mounted on a glass micropipette filled with a saline solution consisting of 150 mM/ l^{-1} NaCl, 10 mM/ l^{-1} KCl, 2 mM/ l^{-1} $CaCl_2$ (Schnuch and Hansen, 1990). Insertion of the micropipette near the area of the hypopharynx allowed exposure of the mouthparts and a proper orientation of the galeal and maxillary palp sensilla. This insect preparation with the ventral side facing up was placed on the reference electrode under a compound microscope. Another glass micropipette, containing the test solution and facing directly opposite to the latter was placed on the recording electrode, and was brought into contact with the sense organ of interest. Action potentials elicited upon contact of the test solution with the sense organ

were recorded. Except for part B of experiment 1 (see results section), all test solutions were applied in random order. Each stimulation was made for about 1 second and a resting period of 3 minutes was allowed between stimulations to prevent adaptation of the sensory cells. All micropipettes containing the test solutions were kept in a moist chamber during each experiment to prevent possible changes of concentration due to the effects of evaporation. A moist chamber consisted of a plastic Petri dish of 100 x 15 mm size containing a circle filter paper moistened with distilled water.

Stimulants

All the carbohydrates and amino acids were obtained from Sigma Chemical Co. (Oakville, ON, Canada). The solutions were dissolved in distilled water containing 25 mMl^{-1} KCl to increase electrical conductance of the solution. The control solution was 25 mMl^{-1} KCl.

Further details of each experiment are found in the results section.

Data Analysis

Electrophysiological responses were obtained using a high impedance (10^{15} Ω) amplifier and recorded as unfiltered DC signals that were then stored onto digital audio tape. The spikes were digitized using the Sapid Tools computer program at a rate of 10,000 points/s for a 1 second period (Smith et al., 1990). All responses were analyzed by counting the number of action potentials within this one second period.

Statistical Analysis

Statistical analyses were done using NCSS (J.L. Hintze, 865 East North, Kaysville, UT, USA). Descriptive statistics were used in all experiments to find means and standard errors. T-tests and ANOVAs were conducted to examine whether or not responses within and between instars differed (for more details see results section).

Results

Experiment 1 (Part A). Preliminary test

Previous electrophysiological studies on detection of carbohydrates by SBW larvae have primarily focused on responses of sixth and fourth instars (Albert and Parisella, 1988*b*; Panzuto and Albert, 1997, 1998; Albert, 2003; Hock, 2005). In a first attempt to obtain electrophysiological recordings from the L1 sensillum on the maxillary palp and the LST on the galea of second instar larvae, four equimolar concentrations (25 mMl^{-1}) of sucrose, fructose, glucose and raffinose were used. The status of the insects was monitored by stimulating the L1 sensillum with sucrose as both the first and the last stimulus in each experiment.

Representative action potentials obtained by stimulations of the L1 and the LST are shown in Figure 1. The spikes are monophasic positive and are similar to those obtained from the LST of fourth and sixth instars (Panzuto and Albert, 1997) and from the L1 sensilla of sixth instars (Albert, 2003).

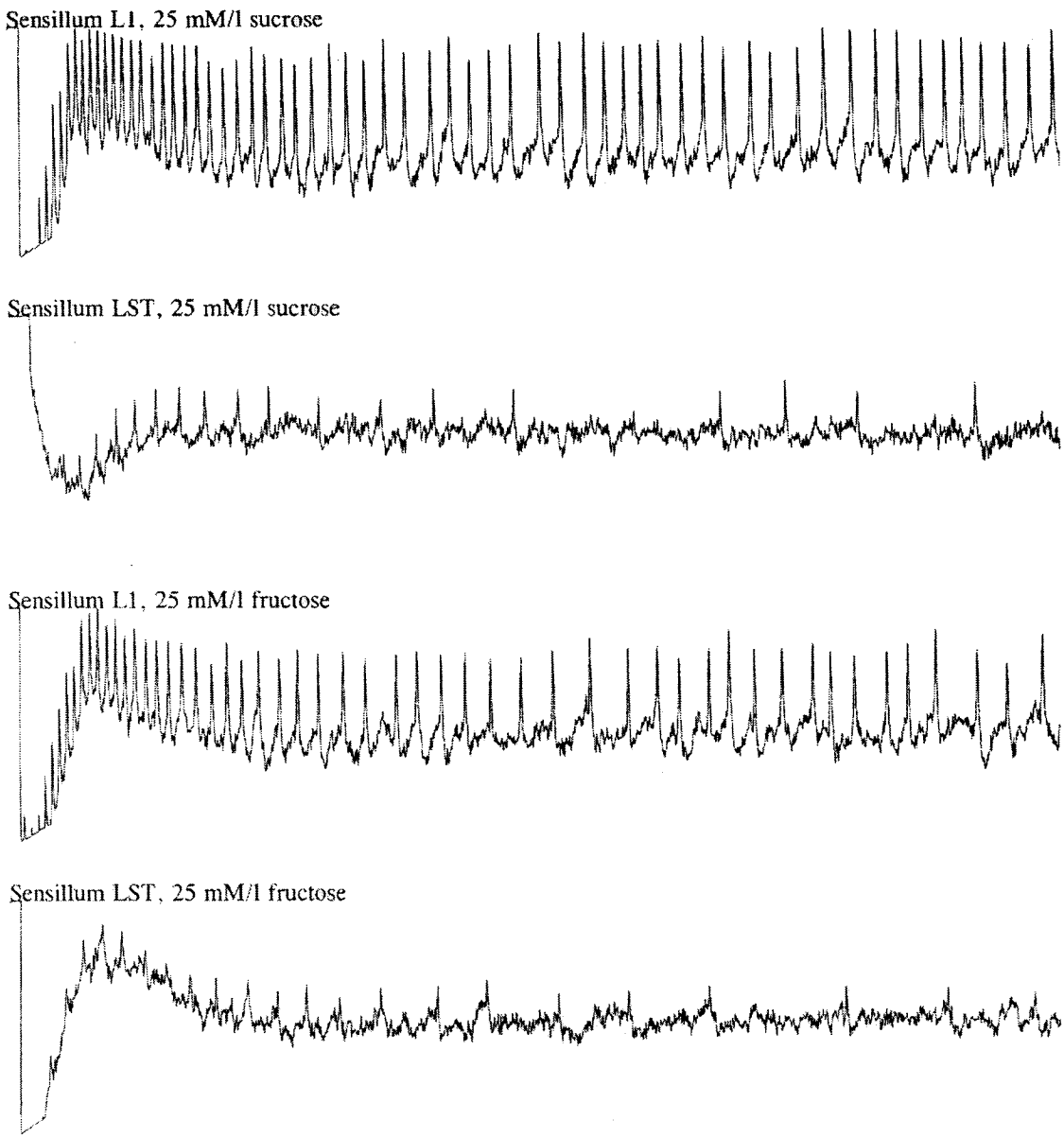

Fig. 1. Electrophysiological traces of the L1 and LST sugar-sensitive cells in response to equimolar concentrations (25 mM l^{-1}) of sucrose and fructose dissolved in 25 mM l^{-1} KCl. All traces are 1 second duration.

Mean impulses/s obtained from L1 and LST by stimulation with carbohydrates are shown in Table 1. A repeated measures two-way Anova comparing the responses between the L1 and the LST revealed that the interaction term was significant ($df = 3, 192$; $F = 18.47$; $p < 0.0001$), suggesting that possible effects of sensilla response are influenced by the carbohydrate type; however such interaction is likely due to the low responses obtained from raffinose and glucose. Low responses to raffinose and glucose were also found by Hock (2005). The author showed that pyranose receptor sites, which bind pyranose sugars such as raffinose and glucose in flies (Shimada, 1987), are not present in the L1 sensillum of sixth instar SBW larvae. Thus, in the present study, spikes obtained from the L1 sensillum by raffinose and glucose stimuli were probably not responses from the sugar cell. These were either from a water cell or leftover sucrose or fructose from previous stimulations as explained by Hock (2005). In the case of the LST, frequencies of responses were not only low to raffinose and glucose but also to fructose and sucrose; thus, suggesting that responses from the sugar-sensitive of the LST of second instar larvae are commonly weak.

A significant effect of sensillum type was observed ($df = 1, 192$; $F = 74.34$; $p < 0.0001$). Responses (mean impulses/s) were higher for the L1 than for the LST. An effect of solution type was also observed ($df = 3, 192$; $F = 37.99$; $p < 0.0001$); sucrose was the most stimulating solution followed by fructose, raffinose and glucose.

A repeated measures two-way Anova, which omitted data obtained from raffinose and glucose stimulations, showed that the interaction effect between sensilla and carbohydrate type was no longer significant ($df = 1, 96$; $F = 2.09$; $p = 0.152$).

Carbohydrates	L1 Mean (S.E ±)	L1 % insects responding	LST Mean (S.E ±)	LST % insects responding
sucrose	38.8 (2.2)	100	10.7 (1.7)	84
fructose	29.4 (3.5)	80	8.2 (1.8)	60
raffinose	7.1 (2.5)	28	7.6 (1.9)	44
glucose	6.7 (2.5)	32	1.6 (0.8)	20

Table 1. Mean impulses/s (± S.E.) of L1 and LST sugar-sensitive cells of the same insects to stimulations with sucrose, fructose, raffinose and glucose (n = 25).

To monitor the status of the insects for any possible deterioration of cell activity during the experiment, the L1 sensillum of each insect was stimulated with 25 mM l^{-1} sucrose at the beginning and at the end of each experiment. The same micropipette containing the test solution was used during the two recording periods, and it was kept in a moist chamber during the intervening time period (about 45 minutes).

A paired *t*-test ($\alpha = 0.05$) comparing the frequency of the sugar-sensitive cell of the L1 sensillum between the initial and the last stimulation showed a significant difference between responses ($t = 4.35$, $n = 25$, $p = 0.0002$). Mean impulses/s varied between the initial and last stimulations, with a higher frequency of firing during the last stimulation (Table 2). This suggests that either evaporation was altering the concentration

of sucrose within the micropipette containing the test solution or that residues from previous tests solutions (sucrose, fructose, raffinose or glucose) were still present at the tip of the palp at the end of the experiment. This would explain the higher number of impulses/s obtained by the last stimulation with sucrose.

Mean impulses/s (S.E ±)	
Initial stimualtion	last stimualtion
38.9 (2.2)	48.6 (2.5)

Table 2. Mean impulses/s (± S.E.) of L1 sugar-sensitive cell response to 25 mMl^{-1} sucrose tested at the initial and the last part of the experiment, (n = 25).

(Part B)

To further investigate the potential temporal source of variability in responses from the L1 sensillum, three stimulations using 25 mMl^{-1} sucrose were made. During the first and second stimulations the same micropipette containing the test solution was used and a 45-minute resting period between recordings was allowed, with the micropipette kept in a moist chamber between stimulations. The third stimulation, 3 minutes following the second one, was done with a fresh micropipette containing the same solution.

If evaporation was the source of variability, then a difference between the first and the second stimulations, with the second eliciting a higher number of impulses/s, was expected; and no difference was expected between the first and the third stimulations. Any evaporation of water between the first and second stimulations would have contributed to an increase in the final concentration of sucrose in the micropipette, and

thus a higher frequency of firing which would not be observed with the third stimulation using a fresh micropipette. However, differences between the first and the second, with the latter eliciting a higher number of impulses/s, and also between the second and the third, again with the latter eliciting a higher number of impulses/s, would be observed in a situation where some leftover sugar residues in or around the tip of the sensillum were the source of the variability.

Paired *t*-tests ($\alpha = 0.05$) revealed that there was a significant difference between responses from the first and second stimulations ($t = 4.33$, n = 9, $p = 0.002$). The number of impulses was higher for the second stimulation compared to the first one (Table 3). However, there was no statistical difference in responses between first and third ($t = 1.38$, n = 9, $p = 0.204$), although the number of impulses/s for the latter were slightly higher, as well as between second and third stimulations ($t = 1.17$, n = 9, $p = 0.277$). This was suggesting that the increase in the number of impulses/s observed during the second stimulation compared to the first one was likely caused by an increase in concentration probably to evaporation.

Mean impulses/s (S.E ±)		
First stimualtion	Second stimualtion	Third stimulation
41 (2.3)	51.7 (3.9)	47.7 (3.3)

Table 3. Mean impulses/s (± S.E.) of L1 sugar-sensitive cell response to three stimulations by 25 mMl^{-1} sucrose tested at different times. (first stimulation = time 0; second stimulation = time 45 min.; third stimulation = time 3 min. after second stimulation), (n = 9). For first and second stimulations the same micropipette was used.

To further investigate the possible effects of evaporation within the micropipette, another experiment was conducted in which amounts of water loss within micropipettes were monitored under two conditions: 1) moist closed chamber positioned next to the recording station and 2) open chamber on the lab bench, directly exposed to air. Changes of meniscus due to water loss were examined at times 4 and 24 hours from the starting point.

	Amount of water loss (nl)			
	closed chamber		open chamber	
pipette #	4 hours	24 hours	4 hours	24 hours
1	0	0	0	446
2	0	0	0	350
3	0	0	0	318
4	0	0	0	308
5	0	0	0	308
6	0	0	0	297
7	0	0	0	308
8	0	0	0	265
9	0	0	0	308
10	0	0	0	297

Table 4. Amount of water loss within micropipettes from both closed and open chambers for two time periods (4 and 24 hours). A sample of 10 micropipettes/chamber was used.

No water loss was observed in any micropipette from either chamber during the first four hours (see Table 4). Small changes were observed after 24 hours only on those micropipettes found within the open chamber. These results suggested that evaporation and changes in concentration of solutes are not factors eliciting an increase in the firing frequency of the sugar-sensitive cell on the L1 between stimulations. This result would

agree with work reported by Hock (2005) in which the presence of leftover sucrose residue on the L1 sensillum of sixth instars influenced subsequent responses to various pyranose sugars.

Experiment 2. L1 sensillum and LST responses to sucrose stimuli on second instars

To further characterize the responses of sugar-sensitive cells from second instar larvae, five concentrations of sucrose (1, 10, 50, 100 and 200 mMl^{-1}) were applied onto the L1 sensillum and the LST.

A repeated measures two-way ANOVA was used to compare whether or not there was an effect between both sensillum and concentration type on responses. A significant interaction between sensillum and concentration type revealed a greater difference in the number of impulses/s between sensilla type at the 10 mM l^{-1} sucrose concentration than at higher concentrations ($df = 4, 210$; $F = 3.15$; $p = 0.015$). A significant effect of sensillum type was observed ($df = 1, 210$; $F = 88.07$; $p < 0.0001$). The L1 sensillum was more sensitive than the LST when stimulated with the same sucrose concentration. Furthermore, concentration type also had an effect on responses ($df = 4, 210$; $F = 87.10$; $p < 0.0001$).

Dose-response curves to a range of sucrose concentrations for the L1 sensillum and for LST are shown in Figure 2. The response thresholds of both sensilla differed. The L1 sensillum is more sensitive than the LST with thresholds of approximately 1 mM l^{-1} and 10 mM l^{-1}, respectively. Cell firing rates increased with increasing concentrations of

sucrose. At first glance, it appeared as if responses from both sense organs level off close to the highest concentration (200 mM l^{-1}). However, the Tuckey-Kramer multiple comparisons test revealed that the responses to 1, 10, 50 and 100 mMl^{-1} all differ from each other except those between 100 and 200 mMl^{-1} sucrose when tested on the L1 sensillum and the LST. Therefore, it appears that responses of both sense organs reach their response plateau at concentrations between 100 and 200 mM l^{-1} sucrose. However, higher concentrations of sucrose would have to be tested in order to ascertain these response plateaus.

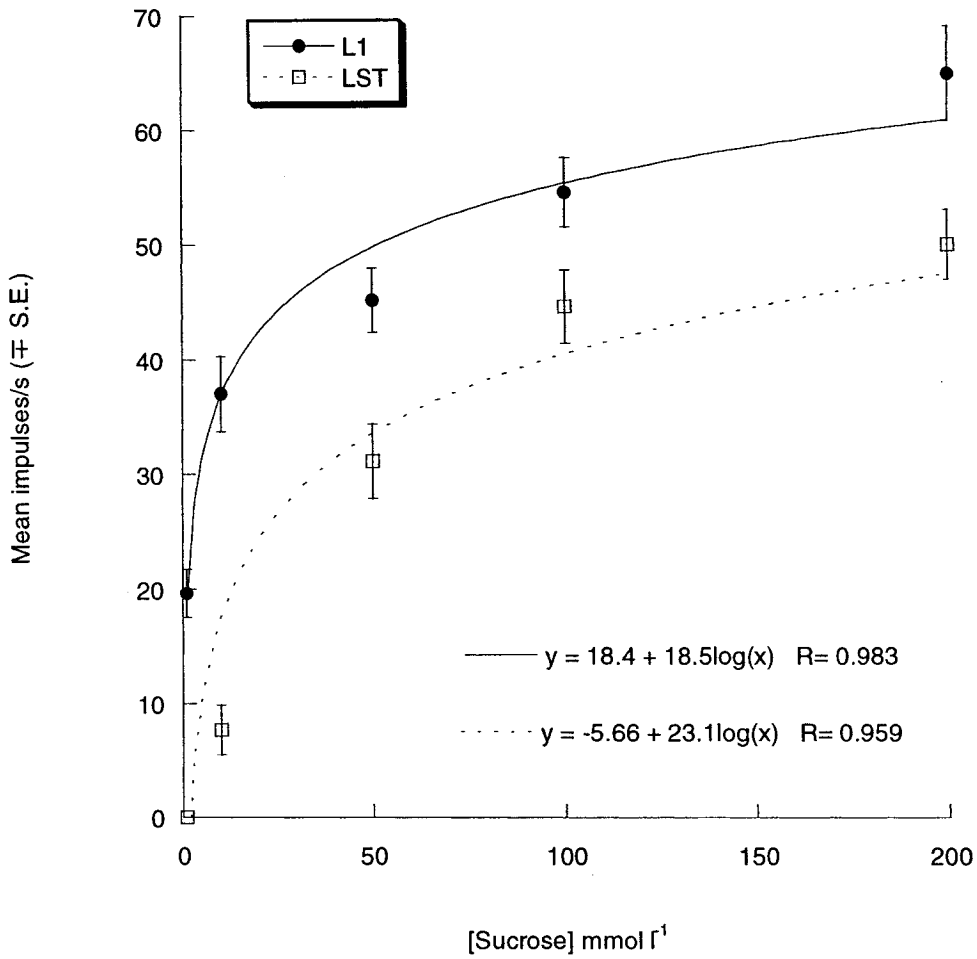

Fig. 2. Responses of L1 sensillum and LST of second instar larvae to increasing sucrose concentration, n = 22 . Responses are in 1000 msec.

From dose response curves such as seen in Figure 2, we can calculate the maximal firing frequency (V_{max}) of the sensory neuron as well as the concentration of the stimulus at which the neuron fires at half-maximal frequency (K_b). These two values are commonly used to express the characteristics of enzyme-substrate interactions and they

are calculated and are obtained by expressing the data as in Figure 2 in a double reciprocal plot.

A double reciprocal (Lineweaver-Burk) plot of concentration (1/ [concentration] (mmol l^{-1})) versus mean impulses/s (1/response) for the L1 sensillum is shown in Figure 3. The Lineweaver-Burk plot is derived from the Michaelis-Menton equation normally used for enzyme kinetics. Enzymatic reactions are similar and comparable to receptor-substrate interactions (Mitchell and Gregory, 1979); thus, a Lineweaver-Burk plot was used for estimating K_b and V_{max} values. The Michaelis constant or K_b estimated value is calculated by solving for the inverse of the x-intercept. The maximal velocity or V_{max} is estimated by solving for the inverse of the y-intercept (Stryer, 2000). The regression equations, V_{max} and K_b values for the first 100, 200, 300, 400, 500 and 1000 ms of response of the L1 sensillum to sucrose stimulation are shown in Table 5. The calculations made using data for the first 100 ms only, and for the first 200, 300, 400 and 500 ms differ since the neuron is adapting quickly. For example, this phenomenon can be seen in Figure 1.

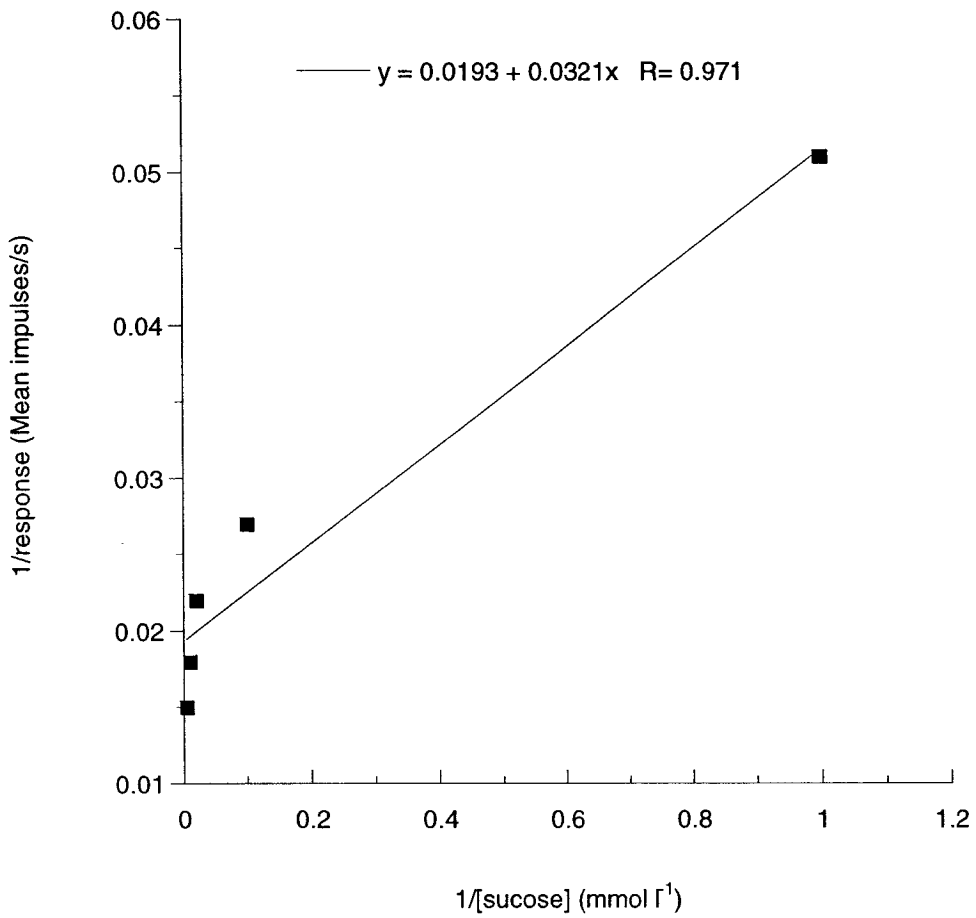

Fig. 3. Double-reciprocal (Lineweaver-Burk) plot of response of the L1 sugar-sensitive cell to sucrose obtained from second instars.

Duration (ms)	Regression equation	V_{max} (impulses/second)	K_b (mmol l^{-1})
100	Y = 0.008X + 0.008	128.5	4.7
200	Y = 0.013X + 0.010	77.2	5.6
300	Y = 0.016X + 0.012	81.4	6.9
400	Y = 0.019X + 0.014	71.6	4.9
500	Y = 0.021X + 0.015	65.9	4.2
1000	Y = 0.032X + 0.019	52.2	1.7

Table 5. Values for regression equation, K_b and V_{max} from double-reciprocal (Lineweaver-Burk) plots of response of the L1 sugar-sensitive cell of second instars for time periods of 100, 200, 300, 400, 500, and 1000 ms.

A Lineweaver-Burk plot was unattainable for the responses of the LST to sucrose. The lowest concentration of the five tested elicited no responses, thus values for K_b and V_{max} were not obtainable.

Experiment 3. L1 sensillum and LST responses to sucrose concentrations on fourth instars

Characterizing responses from sugar sensitive cells of fourth instar spruce budworm larvae have focused on the responses from the LST to some common carbohydrates (Panzuto and Albert, 1997). However, no responses from the L1 sensillum and the LST of fourth instars with varying sucrose concentrations have been attempted up to date. In the present study, seven concentrations (1, 10, 50, 100, 200, 350, and 500 mMl^{-1}) of sucrose were tested on two groups of fourth instar larvae. One group measured responses from the L1 sensillum and the other group responses from the LST.

A two-way Anova was used to determine whether both sensillum and concentration type had an effect on responses (mean impulses/s). No significant

difference was found in responses between the L1 sensillum and the LST ($df = 1, 294$; $F = 0.26$; $p = 0.614$). However, a significant effect of concentration was found ($df = 6, 294$; $F = 50.68$; $p < 0.0001$).

Dose-response curves for the L1 sensillum and for the LST are shown in Figure 4. The number of impulses/s increases in a positive dose-dependant manner with response thresholds of approximately 1 mM l^{-1} for the two sensilla. Tuckey-Kramer multiple comparisons tests revealed that the responses to 1, 10, 50,100 and 200 mM l^{-1} sucrose differ for each sensillum with the exception of 350 and 500 mM l^{-1} for the L1 sensillum and 200 and 350 mM l^{-1} for the LST. It appears that both sense organs might reach their response plateau at concentrations about 350 and 200 mM l^{-1}, respectively. However, higher concentrations of sucrose would have to be tested in order to ascertain these response plateaus.

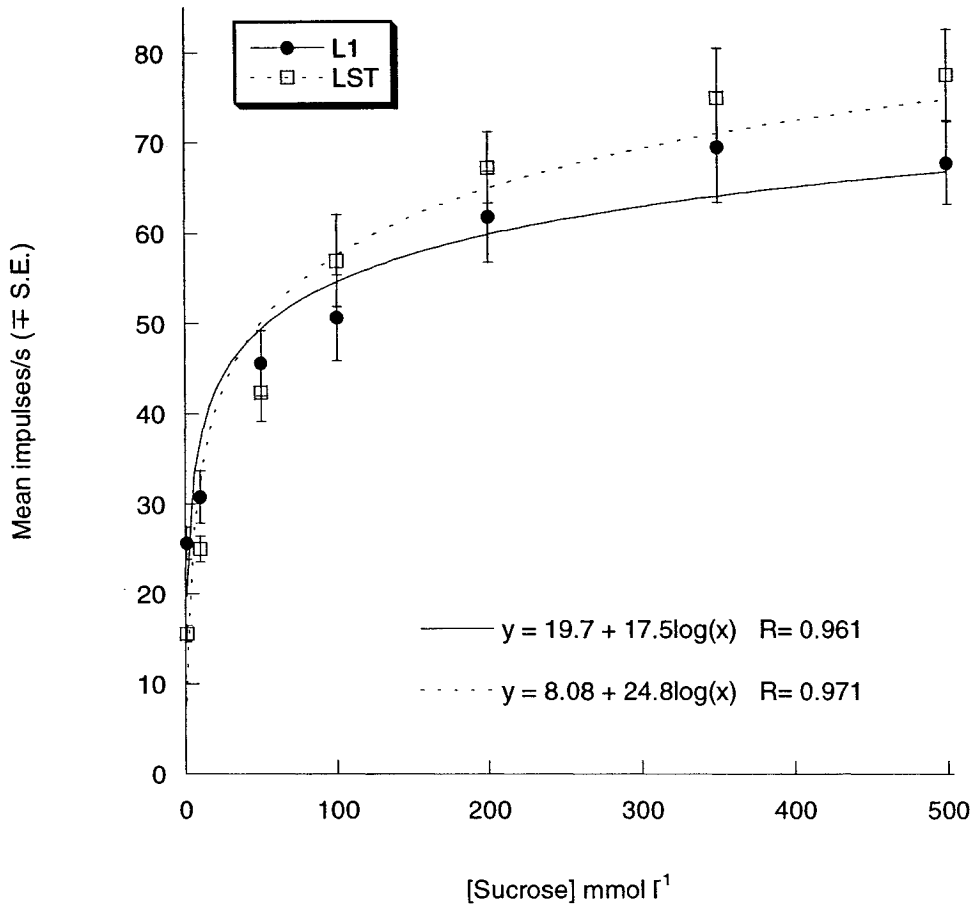

Fig. 4. Responses of L1 sensillum and LST to increasing sucrose concentration obtained from two groups of fourth instars, n = 22 in each group

Values for K_b and V_{max} for the first 100, 200, 300, 400, 500 and 1000 msec. are shown in Table 6a for the L1 sensillum and 6b for the LST. Notice the similar values of V_{max} and K_b for the two sensilla confirming the similarity in responses of the two sense organs.

Duration (ms)	Regression equation	V_{max} (impulses/second)	K_b (mmol l^{-1})
100	Y = 0.009X + 0.007	136.8	6.6
200	Y = 0.012X + 0.010	99.8	4.3
300	Y = 0.013X + 0.012	79.2	1.6
400	Y = 0.015X + 0.015	71	3.7
500	Y = 0.016X + 0.015	65.7	3.1
1000	Y = 0.021X + 0.019	54.8	2.3

Table 6a. Values for regression equation, K_b and V_{max} from double-reciprocal (Lineweaver-Burk) plots of responses of the L1 sugar-sensitive cell of fourth instars for time periods of 100, 200, 300, 400, 500, and 1000 ms.

Duration (ms)	Regression equation	V_{max} (impulses/second)	K_b (mmol l^{-1})
100	Y = 0.019X + 0.008	120.4	5.7
200	Y = 0.024X + 0.010	94.9	4.3
300	Y = 0.028X + 0.013	80.2	3.7
400	Y = 0.030X + 0.014	74.2	8.3
500	Y = 0.032X + 0.015	67.8	6.8
1000	Y = 0.021X + 0.019	58.8	5.2

Table 6b. Values for regression equation, K_b and V_{max} from double-reciprocal (Lineweaver-Burk) plots of responses of the LST sugar-sensitive cell of fourth instars for time periods of 100, 200, 300, 400, 500, and 1000 ms.

Comparisons between L1 sensillum and LST responses to sucrose of second, fourth and sixth instars

Responses between second and fourth instars for each sensillum type were compared using two-way Anovas. No difference was found between instars on responses of the L1 sensillum to a range of sucrose stimuli (df = 1, 210; F = 0.41; p = 0.525). However, responses from the LST were significantly different (df = 1, 210; F = 60.31; p

< 0.0001). These results are consistent with previous work by Albert and Parisella (1988*b*) and Albert (2003). Mean impulses/s during the first 100 and 500 msec. to stimulation by a range of sucrose concentrations for second, fourth, and sixth instar are shown in Table 7a and 7b, respectively (data from sixth instar obtained from Albert and Parisella, 1988*b*; and Albert, 2003). Note the rapid drop in mean impulses/s for the first 100 to 500 msec. as a result of fast adaptation rate of the neurons. Comparing mean impulses/s for each concentration between the three larval stages shows similar values for the L1 sensilla. However, some larger differences between mean impulses/s can be noted between instars for the LST.

Sucrose (mM)	L1 Mean impulses/s (S.E. ±)			LST Mean impulses/s (S.E. ±)		
	2nd instar	4th instar	6th instar	2nd instar	4th instar	6th instar
0.5	-	-	-	-	-	46 (5.7)
1	62 (8.7)	64 (3.9)	12 (11.8)	0	38 (1.9)	110 (13.6)
5	-	-	-	-	-	144 (13.6)
10	98 (8.7)	91 (8.2)	70 (8.5)	21 (6.0)	61 (3.0)	167 (14.6)
50	136 (3.5)	120 (5.3)	143 (13.7)	82 (6.1)	113 (5.9)	177 (11.1)
100	121 (7.3)	139 (9.4)	154 (12.5)	98 (6.7)	132 (6.3)	180 (16.3)
200	150 (5.7)	153 (9.6)	160 (10.1)	110 (5.9)	149 (5.5)	-
350	-	166 (12.0)	167 (19.0)	-	150 (7.9)	-
500	-	155 (9.6)	178 (16.9)	-	152 (7.2)	-

Table 7a. Mean impulses/s (± S.E.) during the first 100 msec. of L1 sensillum and LST sugar-sensitive cells responses to stimulation with various sucrose concentration for second, fourth and sixth instars spruce budworm larvae. Data for sixth instar on LST and L1 sensillum response to sucrose from Albert and Parisella (1988*b*); and Albert (2003).

Sucrose (mM)	L1 Mean impulses/s (S.E. ±)			LST Mean impulses/s (S.E. ±)		
	2nd instar	4th instar	6th instar	2nd instar	4th instar	6th instar
0.5	-	-	-	-	-	23 (2.3)
1	28 (3.9)	33 (1.8)	4 (2.3)	0	22 (1.2)	49 (5.0)
5	-	-	-	-	-	79 (9.2)
10	50 (4.3)	41 (3.7)	33 (3.4)	12 (3.4)	34 (1.7)	89 (11.3)
50	60 (3.3)	57 (4.2)	64 (5.6)	44 (3.6)	57 (3.8)	101 (10.4)
100	69 (4.1)	65 (5.9)	77 (7.9)	59 (3.7)	72 (5.7)	98 (14.5)
200	81 (4.4)	76 (6.4)	84 (5.2)	70 (3.8)	84 (4.1)	-
350	-	85 (7.5)	92 (8.1)	-	91 (6.0)	-
500	-	82 (5.7)	106 (8.8)	-	93 (5.2)	-

Table 7b. Mean impulses/s (± S.E.) during the first 500 msec. of L1 and LST sugar-sensitive cells responses to stimulation with various sucrose concentration for second, fourth and sixth instars spruce budworm larvae. Data for sixth instar on LST and L1 sensillum response to sucrose from Albert and Parisella (1988*b*); and Albert (2003).

A summary of the V_{max}, K_b, threshold and plateau values of responses from the L1 sensillum and LST to sucrose in second, fourth and sixth instars is shown in Table 8. The L1 has a threshold that remains constant through the three larval stages with increasing K_b values with larval age. However, notice the decrease in threshold and K_b values with larval age for the LST, suggesting an increase in sensitivity and increases in binding affinity between substrate-receptors complexes.

Instar	Sensillum	V_{max} (100msec.) (impulses/s)	V_{max} (500msec.) (impulses/s)	K_b (mmol l^{-1})	Threshold (mmol l^{-1})	Plateau (mmol l^{-1})
Second	L1	128.5	65.9	4.7-4.2	1	> 200
	LST	N/A	N/A	N/A	10	> 200
Fourth	L1	136.8	65.7	6.6-3.1	1	350
	LST	120.4	67.8	5.7-6.8	1	200
Sixth	L1	200	105	25-25	1 – 10	200
	LST	201	110	1.5-1.7	<0.5	50

Table 8. Response characteristics (V_{max}, K_b, threshold and plateau values) of L1 sensillum and LST of second, fourth and sixth instar spruce budworm larvae to the given concentrations of sucrose. Data for sixth instar on LST and L1 sensillum response to sucrose from Albert and Parisella (1988*b*); and Albert (2003).

Experiment 4. Characterization of amino acid cells of second instars

According to Panzuto and Albert (1998) the LST on the galea of SBW larvae bear an amino-acid sensitive cell that differs in sensitivity of responses to various amino acids between fourth and sixth instars, with the latter showing greater sensitivity. In the present study the same l-amino acids used by Panzuto and Albert (1998) were used to characterize responses from the amino acid-sensitive cell in the LST of second instar larvae. In addition, the L2 sensillum on the maxillary palp was also tested as no information is available to date from this sensillum except for some preliminary data (Albert, unpublished).

Table 9 shows mean impulses/s obtained from stimulation of the LST with various l-amino acids; concentrations of each amino acid were used as in Panzuto and

Albert (1998) in which each concentration was chosen as representative of those occurring naturally in balsam fir and white spruce according to Kimmins (1971).

Amino Acid	Concentration (mM l^{-1})	Mean impulses/s (± S.E.) LST	Sample size LST
l-proline	50	-	-
l-glutamic acid	2	0.5 (0.45)	20
l-valine	20	11 (3.9)	11
l-leucine	50	29.5 (6)	10
l-histidine	30	0	9
l-cystine	0.5	0	9
l-tyrosine	2.5	0	9
l-glycine	10	0	9
l-serine	50	0	9
l-threonine	50	0.3 (0.3)	15
l-aspartic acid	20	11.6 (4.6)	10
l-lysine	50	0	15
l-alanine	100	1.5 (1.1)	15
l-arginine	100	4.9 (1.7)	10

Table 9. Mean impulses/s (± S.E.) of amino-acid cell in the LST of second instar larvae to stimulation with various l-amino acids dissolved in 25 mM l^{-1} KCl. Sample size range between 9-20 insects.

The highest response rate from the LST were obtained by l-leucine, l-aspartic acid and l-valine. The large standard errors suggest a high degree of variability in responses. The spikes were monophasic-positive and of medium size (not shown), are thus similar to those described by Panzuto and Albert (1998) for fourth and sixth instars. These results, in addition to a lack of response from the control solution (25 mM l^{-1} KCl), confirm the presence of an amino-acid sensitive cell on the LST of second instars.

Table 10 shows mean impulses/s for responses from stimulations of the LST with various l-amino acids for second, fourth and sixth instars (data for fourth and sixth instar

obtained from Panzuto, 1996). Comparing the pattern of responses from the three different instars we observe that the number of impulses/s of the amino acid sensitive-cell of the LST increases for most amino acids as larvae grow, with the exception of l-tyrosine, l-threonine, l-lysine and l-alanine.

Amino Acid	Concentration ($mM\ l^{-1}$)	(Mean ± S.E.) Second instars	Second instars n	(Mean ± S.E.) Fourth instars	Fourth instars n	(Mean ± S.E.) Sixth instars	Sixth instars n
l-glutamic acid	2	0.5 (0.45)	20	62 (5.4)	13	122 (7.2)	15
l-valine	20	11 (3.9)	11	105 (7.5)	19	110 (7.0)	19
l-leucine	50	29.5 (6)	10	82 (7.3)	16	105 (7.2)	18
l-histidine	30	0	9	42 (8.8)	17	83 (12)	12
l-cystine	0.5	0	9	49 (5.7)	17	78 (9.1)	18
l-tyrosine	2.5	0	9	89 (10.1)	15	72 (11.5)	12
l-glycine	10	0	9	43 (6.7)	13	68 (10.8)	12
l-serine	50	0	9	28 (3.7)	16	67 (5.9)	17
l-threonine	50	0.3 (0.3)	15	62 (9.4)	18	59 (6.2)	12
l-aspartic acid	20	11.6 (4.6)	10	29 (3.3)	14	53 (7.2)	13
l-lysine	50	0	15	74 (11.0)	17	47 (5.5)	13
l-alanine	100	1.5 (1.1)	15	63 (8.3)	17	43 (5.6)	13
l-arginine	100	4.9 (1.7)	10	0	-	0	-

Table 10. Mean impulses/s (± S.E.) for the first 1000 msec. of the amino acid-sensitive cell in the LST to stimulations with various amino acids for second, fourth and sixth instars spruce budworm larvae. (Data for fourth and sixth instar on LST responses to amino acid were obtained from Panzuto, 1996).

Out of the fourteen amino acids tested on the L2 sensilla, l-arginine, l-proline, l-cystine and l-tyrosine elicited the highest responses, with mean cell firing rates of 33 impulses/s for the first two amino acids and 16 impulses/s for the remaining two (Table 11). Representative action potentials are shown in Figure 5. Although such responses suggested the potential presence of an amino acid-sensitive cell, further investigation was necessary since previous data obtained from preliminary tests on the L2 sensillum of sixth instar revealed the presence of a water-sensitive cell (Albert, unpublished). Thus, to rule out the possibility that responses obtained in the present test were from a water or

KCl-sensitive cell, distilled water and varying concentrations of KCl (5, 10, 25, 50, 100, and 200 mM l^{-1}) were applied onto the L2 sensilla of second instar larvae.

Amino Acid	Concentration (mM l^{-1})	Mean impulses/s (± S.E.) L2	Sample size L2
l-proline	50	33.7 (2.8)	13
l-glutamic acid	2	0.7 (0.7)	9
l-valine	20	0.8 (0.8)	14
l-leucine	50	2.1 (1)	19
l-histidine	30	1.2 (1.2)	9
l-cystine	0.5	16.7 (1.7)	9
l-tyrosine	2.5	16.3 (1.8)	9
l-glycine	10	2 (1.4)	9
l-serine	50	0.2 (0.2)	20
l-threonine	50	4.2 (2.1)	19
l-aspartic acid	20	6.2 (2.1)	14
l-lysine	50	0	15
l-alanine	100	5.5 (2)	19
l-arginine	100	32.1 (6.7)	14

Table 11. Mean impulses/s (± S.E.) of amino-acid cell in the L2 sensillum of second instar larvae to stimulation with various l-amino acids dissolved in 25 mM l^{-1} KCl. Sample size range between 9-20 insects.

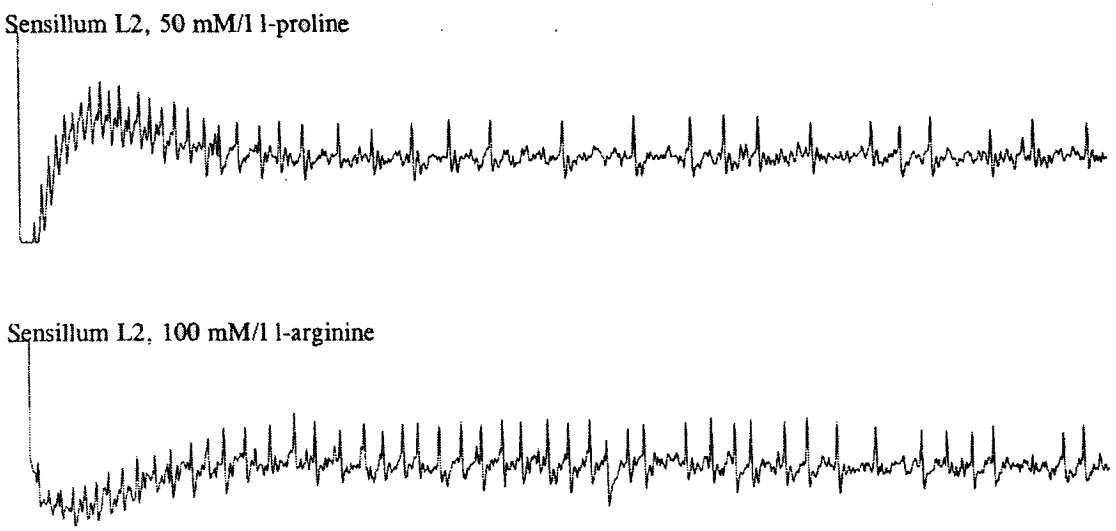

Fig. 5. Responses from the L2 sensillum of second instars to stimulations with 50 mM l^{-1} l-proline and 100 mM l^{-1} l-arginine.

Action potentials from water and KCl stimulation are shown in Figure 6. Responses to distilled water from the L2 sensillum of second instars had a mean of 14.5 (± 5.1) impulses/s from a sample of 17 insects. Stimulations with increasing concentrations of KCl showed no activity other than that from the water cell (see Table 12). Note the decrease in the mean number of impulses/s at the highest KCl concentrations suggesting the inhibition of the water cell beyond the 100 mM KCl concentration. These results are consistent with the presence of a water-sensitive cell and the absence of a KCl-sensitive cell in the L2 sensillum.

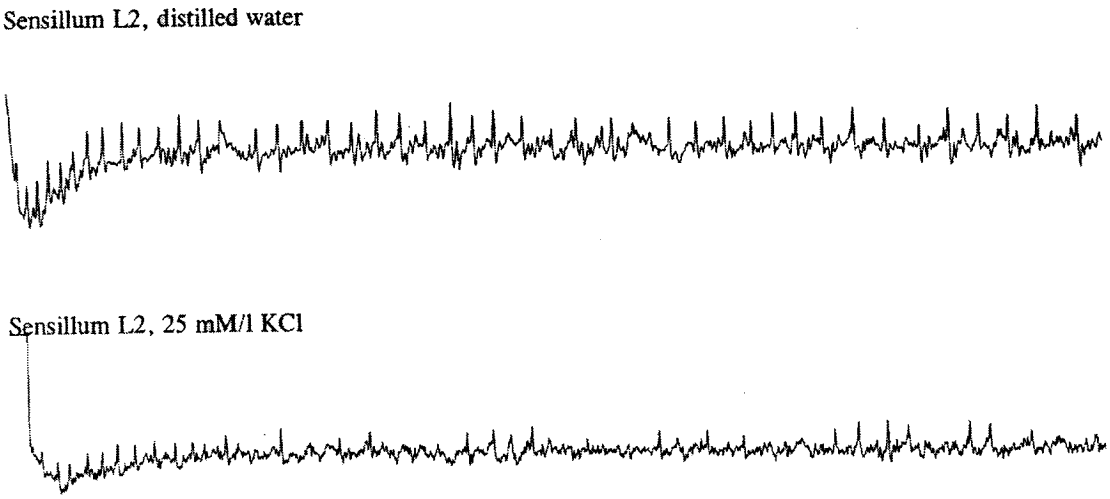

Fig 6. Responses from the L2 sensillum of second instars to stimulations with distilled water and 25 mM l^{-1} KCl.

KCl concentrations n = 10	Mean impulses/s (± S.E.) L2
5	12.5 (4.7)
10	13.0 (2.6)
25	9.1 (4.4)
50	19.8 (4.6)
100	4.2 (5.6)
200	6.0 (2.5)

Table 12. Mean impulses/s (± S.E.) from the L2 sensillum of second instars stimulated with increasing concentrations of KCl.

To investigate further whether the responses obtained from the L2 sensilla were a result of amino acid stimulation, various concentrations of l-proline (0.5, 1, 10, 50 and 100 mM l^{-1}) dissolved in either 25 or 5 mM l^{-1} KCl were used to stimulate the respective sensilla.

Responses (mean impulses/s) of L2 sensilla to increasing l-proline concentrations are shown in Table 13.

	Mean impulses/s (± S.E.) L2 sensillum	
Proline concentration (mM l^{-1})	(25 mM l^{-1} KCl); n = 8	(5 mM l^{-1} KCl); n = 5
0.5	23.3 (2.6)	33.4 (7.8)
1	23.6 (4.0)	24.8 (3.6)
10	28.8 (2.4)	32.2 (3.7)
50	30.8 (3.6)	38.2 (3.8)
100	28.6 (1.3)	36.2 (3.6)

Table 13. Mean impulses/s (± S.E.) from the L2 sensillum from 2 groups of second instars stimulated with various l-proline concentrations at either 25 or 5 mM l^{-1} KCl.

Responses ranging between 23.3 and 38.2 impulses/s as concentrations of l-proline were increased revealed the lack of activity from an amino acid cell on the L2 sensilla. A two way Anova revealed that there are no significant differences between l-proline concentrations ($df = 4, 55$; $F = 2.23$; $p = 0.077$). However, a significant effect was observed between the two KCl concentrations (5 and 25 mM l^{-1}) ($df = 1, 55$; $F = 6.26$; $p = 0.015$) and there was no interaction between l-proline and KCl concentrations ($df = 4, 55$; $F = 0.45$; $p < 0.772$). The significant effect of KCl could be explained by an inhibitory effect of the water cell responses as KCl concentrations are increased. This is observed

by the slightly lower number of impulses/s elicited by 25 mM l^{-1} KCl concentration for all l-proline concentrations compared to those of 5 mM l^{-1} KCl concentration. These results suggest that responses obtained by stimulations of the L2 sensilla with amino acids were responses from the water cell and not from an amino acid-sensitive cell.

Experiment 5. MST responses to l-proline stimuli on second instars

An interesting role of the proline-sensitive cell in the MST on the galea of fourth and sixth instars has been reported in relation to a possible function as detector of water stress. That is due to its uniqueness in responding only to l-proline, known to increase during periods of plant stress (Zwiazek and Blake, 1990), and not to any other amino acid (Panzuto and Albert, 1998). In order to determine the responsiveness of the MST of younger larvae, seven concentrations of l-proline were tested on second instars (0.001, 0.01, 0.1, 1, 10, 50 and 100 mM l^{-1}).

A dose-response-curve to increasing l-proline concentration on the MST is shown in Figure 7. Stimulus concentrations below 0.1 mM l^{-1} elicited no responses from the l-proline-sensitive cell; however, beyond this point the cell firing frequency increased with increasing concentrations of the l-proline. A paired *t*-test ($P < 0.05$) was used to determine at what concentration the cell reaches saturation. A significant difference between concentrations of 0.1 and 1 and 1 and 10 mM l^{-1} l-proline was observed. However, no difference between 10 and 50 mM l^{-1} l-proline was evident ($t = -0.89$, $n = 13$, $p = 0.13$) suggesting that the cell's response reaches a plateau beyond the 10 mM l^{-1} concentration.

A one-way Anova revealed that there was a concentration effect (df = 6, 84; F = 59.20; $p < 0.0001$). The Tuckey-Kramer multiple comparisons test revealed that the responses to 0.1, 1, and 10 mM l^{-1} l-proline all differ from each other except those between 10 and 50 and 50 and 100 mMl^{-1}. Therefore, it appears that responses of the MST sensillum reached their response plateau at concentrations between 100 and 200 mM l^{-1} l-proline. However, higher concentrations of l-proline would have to be tested in order to ascertain this response plateau.

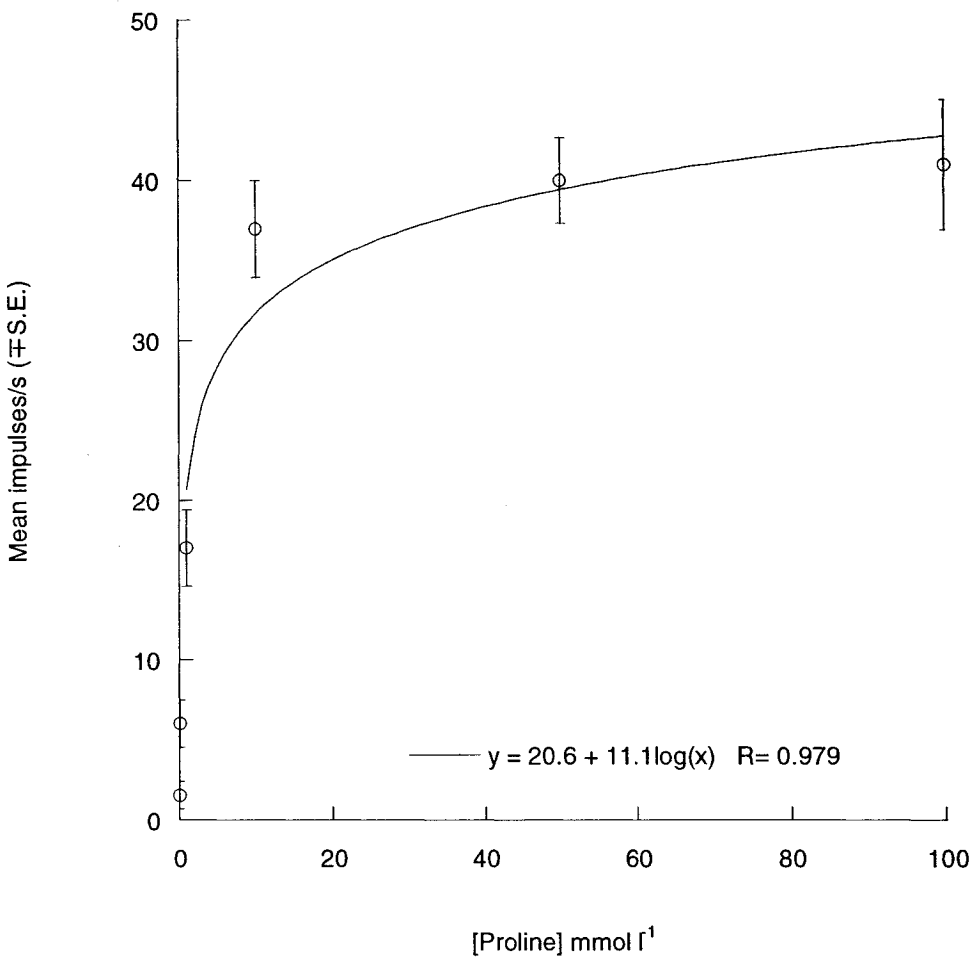

Fig.7. Responses from MST to increasing l-proline concentration obtained from second instars (n = 13).

Comparisons of second, fourth and sixth instars' MST responses to l-proline

Table 14 shows values of V_{max}, K_b, threshold and plateau of responses to stimulation by l-proline in the MST of second, fourth and sixth instar larvae (values for fourth and sixth instars were obtained from Panzuto, 1996). An increase in sensitivity

with larval age is illustrated by a decrease in K_b and threshold values. An increase in the plateau between 10 to 50 mM l^{-1} from second to fourth instar demonstrates a higher capacity of response of the MST of older larvae.

Instar	V_{max} (impulses/s)	K_b (mmol l-1)	Threshold (mmol l-1)	Plateau (mmol l-1)
Second	22.2	0.1	> 0.1	10
Fourth	111.1	0.1	0.001	50
Sixth	46.5	0.001	0.001	50

Table 14. Response characteristics (V_{max}, K_b, threshold and plateau values) of MST of second, fourth and sixth instar spruce budworm larvae to the given concentrations of l-proline (data for fourth and sixth instar from Panzuto, 1996). V_{max} calculated from 1 second traces.

DISCUSSION

Electrophysiological recordings were obtained from various sensilla on the mouthparts of SBW second instar larvae, the first such study on individual gustatory sensilla of very young larvae of phytophagous lepidoptera. Until now, electrophysiological studies have focused on the responses from chemoreceptor sensilla of older larvae (Albert and Parisella, 1988*b*; Schoonhoven et al., 1990; De Boer, 1991; Panzuto and Albert, 1998). This is undoubtedly related to the larger size of older instars, which makes electrophysiological testing more feasible, as well as to the fact that these usually cause the greatest damage to host plants, making them the principal target when developing pest control strategies (Retnakaran et al., 1999).

Studies with fourth and sixth instar SBW larvae have provided valuable information in relation to changes in larval feeding preferences occurring during the period of larval growth (Harvey, 1974, Albert and Bauce, 1994, Guertin and Albert, 1994). These studies emphasize the idea that changes in feeding preferences parallel the nutritional needs of caterpillars by demonstrating that younger (fourth) instars have greater preference for nitrogenous compounds while older (sixth) instars prefer carbohydrates (Harvey, 1974; Albert and Bauce, 1994). The object of the present study was to compare physiological responses from second instars with those from older instars to determine if the pattern of changes observed between responses from fourth and sixth instars extend also to the second instars.

In the present study, I found that the galeas' LST of second instar larvae are less sensitive to varying sucrose concentrations compared to older larvae. These results support the physiological study conducted by Panzuto and Albert (1997) where responses to sucrose from the LST of SBW fourth and sixth instar larvae displayed an age-dependant sensitivity that increased with larval age. The result from the present study and that of Panzuto and Albert (1997) demonstrate that responses to sucrose from the sugar-sensitive cell in the LST of SBW larvae correlates well with larval growth and the feeding needs of the larvae; as larvae grow, their gustatory responses to sucrose increase. Sucrose is a strong phagostimulant (Heron, 1965) and in the SBW its preference over other carbohydrates has been confirmed (Albert et al., 1982). Evidence indicates that sixth instar SBW larvae prefer feeding from sucrose solutions that induce the highest cell firing frequency (Albert and Parisella, 1988*b*). Older instars, preparing for metamorphosis, require a higher amount of energy reserves that are obtained in the form of carbohydrates. Therefore, it would be advantageous for the older larvae to have a greater capacity for detection of sucrose compared to the younger larvae, since the latter do not have the same energy requirements.

Comparisons between the neural responses to sucrose between second, fourth and sixth instars in both the LST (present study; Panzuto and Albert, 1997) and L1 sensilla (present study; Albert, 2003) revealed an interesting pattern in the response characteristics of the two neurons. The sugar-sensitive cell in the L1 sensillum exhibits a higher frequency of response than that of the LST when stimulated with similar sucrose concentrations. Responses from the sugar cell in the LST, as previously mentioned, significantly increase with increasing larval age; however, in the L1 sensillum, no major

differences are observed in the number of impulses/s elicited by the sugar cell between the different instars when stimulated with similar sucrose concentrations. This indicates that the L1 sugar-sensitive neuron is more sensitive than that in the LST, and that few changes, if any, occur in this sensillum in terms of response characteristics through larval development. Variability between chemoreceptor neurons in different sensilla has been documented in other caterpillars. In *Pieris brassicae,* one cell responsive to sucrose has been identified in three different sensilla; one in the epipharynx and the other two in the LST and the MST. Two of these cells differ in their response characteristics, which may suggest different functions, and ablation experiments indicate that the inputs from the three chemosensory sensilla are essential for normal feeding to occur (Blom, 1978). In SBW larvae, an indication that both LST and L1 sensilla have different roles has been suggested on the basis of their response characteristics observed in sixth instar larvae; however, the assumption that both sensilla are necessary for proper feeding to occur remains to be investigated.

Analysis of the sucrose content of one-year-old needles of balsam fir at the time second instars are feeding is approximately 100 mM l^{-1} (Little, 1970). This sucrose level falls within the range of detection for both LST and L1 sensilla of second instars having thresholds of 10 mM l^{-1} and 1 mM l^{-1} respectively, and saturation levels > 200 mM l^{-1} for both sensilla. Similarly, fourth instars, which prefer feeding from current year needles, are able to detect the approximately 40 mM l^{-1} sucrose content of balsam fir (Little, 1970) indicated by thresholds of 1 mM l^{-1} and plateau levels of 200 and 350 mM l^{-1} for the LST and the L1 sensilla respectively. These results suggest some built-in redundancy in the gustatory system, with two cells responding to sucrose in two separate sensilla of the two

larval instars. Albert (2003) hypothesized that the sugar-sensitive neuron in the L1 sensillum of sixth instars might possibly serve to "detect higher-than-normal levels of sucrose or other sugars in its host plants". The L1 sensillum of sixth instars has a threshold that falls between 1 and 10 mM l^{-1} with plateau of 200 mM l^{-1} (Albert, 2003). Thus, since responses from the LST from sixth instar larvae respond maximally to a concentration of 50 mM l^{-1} sucrose, it would seem advantageous for the larvae to have a second cell responding to higher concentrations of sucrose as does the L1 sensillum in sixth instars, particularly if the concentration of carbohydrates increases in the host plant, as it does during periods of plant stress (Zwiazek and Blake, 1990). What the present study and that of Albert (2003) indicate is that although a built-in redundancy is observed between responses of the sugar-sensitive cell of both LST and L1 sensilla to sucrose on second and fourth instars, differences do exist in the response characteristics of these two sensilla in the oldest larvae. One can speculate that these chemosensory organs possess some plasticity as reflected by their variability in sensorial responses from one instar to the other. However, for the time being no clear inferences can be made about the mechanisms modulating these differences in chemoreceptor sensitivity between the fourth through the sixth larval instars.

Electrophysiological responses obtained by stimulation of the amino acid cell of the LST in second instar larvae revealed that only l-leucine, l-aspartic acid and l-valine induce a response in this cell. This indicates that the LST of second instars is not very responsive to amino acids as only three of thirteen amino acids tested elicited a response. In addition, the amino acid cell's firing frequency to these amino acids is much lower for second instars, with average responses between 11 and 29.5 impulses/s compared to

fourth and sixth instars where they are between 29 and 110 impulses/s (Panzuto and Albert, 1998). This increase in sensitivity observed from younger to older instars agrees with the work of Panzuto and Albert (1998) where fourth instars displayed a lower sensitivity to most amino acids compared to sixth instars. Behavioural studies conducted on sixth instars demonstrated that l-proline, l-serine, l-alanine, and l-lysine have a stimulating effect, while l-valine has a deterrent one, and the remaining amino acids have no effect (Albert and Parisella, 1988). This order of behavioural preference does not correlate with the firing frequency obtained from either sixth instar (glutamic acid > valine > and leucine), fourth instar (valine > tyrosine and > leucine) (Panzuto and Albert, 1998) or second instars. However, an interesting feature observed is that although the order of responsiveness varies between the three instars, leucine and valine, the latter as already mentioned a behavioural deterrent, are among the most electrophysiologically stimulating amino acids for the SBW. This could suggest that responses from these two amino acids by the LST may be important for the SBW. During behavioural tests, a number of sensilla make contact with the substrate and the final output is a summation of various chemosensory responses from many sensilla. This suggests that although the LST can respond to amino acids stimulation, other sensilla may be involved in mediating the responses to these compounds which could suggest an across pattern type of coding (Schoonhoven and Van Loon, 2002). In larvae of the Colorado potato beetle *Leptinotarsa decemlineata,* at least six sensory cells in the mouthparts are sensitive to some amino acids (Mitchell, 1974). Therefore, solid assumptions about the detection of amino acids by SBW larvae cannot be made until our knowledge of all the other

chemosensory cells, including those found in the epipharynx and the maxillary palpi, is more detailed than at present.

Stimulations with l-amino acids confirmed that the L2 sensillum of second instars does not bear an amino acid cell, however it does contain a water-sensitive cell. This corroborates previous results obtained from sixth instars suggesting the presence of a water cell in this sensillum (Albert, unpublished). The water response remains constant when KCl is dissolved in water at concentrations lower than 100 mM l^{-1}; however, beyond this salt concentration the water response becomes inhibited. Similar response characteristics have been documented in the taste sensilla of the blowfly *Phormia regina* (Evans and Mellon, 1962) and in *Bombyx mori* (Ishikawa, 1976) where water cells exposed to increasing salt concentrations are increasingly inhibited and may even become fully suppressed. The authors suggest that sugars and amino acids may also inhibit the water cells' responses. A similar inhibition of the water cell was noted in the present study with stimulations of l-proline concentrations at 25 mM l^{-1} KCl. The significance of these inhibitions is not yet understood. However, these interactions occurring with the water cell and KCl raise some interesting questions: are these interactions unique to water cells? Do other mixtures of solutions induce similar responses in other chemoreceptor sensilla on the SBW and/or other caterpillars? To address these questions more electrophysiological testing using single and mixtures of compounds are required.

Like the previous results obtained from sucrose and amino acids, second instar responses from the MST to stimulations with l-proline display a pattern of cell firing frequency that is lower than those noted for fourth and sixth instars by Panzuto and Albert (1998). In addition, this l-proline-sensitive cell has a higher threshold and lower

saturation level than the corresponding cell in both the fourth and the sixth instars (Panzuto and Albert, 1998). This implies that early instars are less sensitive to l-proline than older ones. Panzuto and Albert (1998) inferred that the l-proline-sensitive cell in the MST could signal important information to the insect about the nutritional status of the plant. Proline is not considered an essential amino acid for growth (Schoonhoven and Van Loon, 2002); nevertheless, it is known to stimulate feeding (Heron, 1965). Since sixth instars are the largest and the most long-lived larval stadium of the SBW (McGugan, 1954), the amount of food consumed by the older larvae is much greater than that consumed by earlier instars. In the larvae of *Helicoverpa armigera,* the total amount of food consumed by the fifth instars is about 3.5 times more than that consumed by the fourth instars (Browne and Raubenheimer, 2003). In most insects there is a positive relation between phagostimulants and feeding behaviour with the responses from chemoreceptors (Blom, 1978). Therefore, having a higher capacity of detection not only of l-proline but of other compounds such as sucrose for older instars could be an adaptation to maximize the amount of food consumed by larvae that are close to reaching pupation.

In summary, differences in chemosensory responses are observed between instars and these might reflect potential changing nutritional requirements of the insect. This is observed in responses from the LST to stimulations with sucrose solutions where there is an age-dependent response. The role of the L1 sensillum as a stressed plant detector remains valid for sixth instars, but it is not so evident for younger instars. However, since the L1 sensillum is capable of responding to concentrations of sucrose found within the host plant, as does the lateral styloconic sensillum, it is possible that this sense organ

plays an important role in feeding during larval growth. Furthermore, the responses to amino acids observed from second instars do not conform with the current behavioural data in which early instars prefer consuming the nitrogen rich extracts of balsam fir needles compared to older instars that prefer the extracts from carbohydrate rich needles (Bauce and Albert, 1994). However, no direct comparisons can be made with the study of Bauce and Albert (1994) since they used fourth and sixth instars while second instars were used in the present study. In addition, I only used single amino acids as test compounds whereas in the behavioural study extracts of balsam fir needles were used, and these contained a variety of chemicals. If younger instars have a higher requirement for nitrogenous compounds than older larvae then it is probably safe to assume that other chemosensilla in combination with the LST and MST could be involved in mediating the detection of nitrogenous compounds. However, in order to make a more concise statement about how younger larvae satisfy their nitrogen requirements further investigation is necessary.

In order to complement what is known to date, future research should aim to gain a better understanding of the importance of both LST and L1 sensilla for normal feeding by SBW larvae. To do this, studies could be done by removing specific sense organs and by comparing the behavioural responses of the larva with different combinations of intact and ablated sensilla. Due to the small size of second instars SBW larvae this may be very challenging; however, it might be feasible using sixth instars. Moreover, further electrophysiological testing is required on the chemosensory organs of the maxillary palp such as the L2 and the A3 sensilla, and in the epipharynx in order to identify their individual response characteristics, which in turn will provide a better insight on the

chemosensory capacity of the larvae. Finally, conducting electrophysiological experiments using single and mixtures of compounds could give a more realistic sense of interactions occurring at the level of chemoreceptors since, in nature, insects encounter not one but an array of chemical compounds in different combinations.

To have a better understanding of how insects make their feeding decisions and the mechanisms involved in these processes, it is critical to have a good knowledge of the physiological aspects underlying these desicions. Noting that physiological differences exist between larval instars is an important finding and implies that care should be taken when interpreting and generalizing studies conducted with older instars. In addition, since this is the first step in understanding sensorial responses from early instars, future research along this line could contribute to developing pest control strategies that could be applied at early stages of larval development, thus diminishing potential tree damage that occurs during SBW outbreaks.

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