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FEEDING AND OVIPOSITION BEHAVIOR OF TOBACCO HORNWORMS, *MANDUCA SEXTA*, IN RELATION TO *MYO*-INOSITOL

by

Nancy Marie Nelson

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DEPARTMENT OF ENTOMOLOGY

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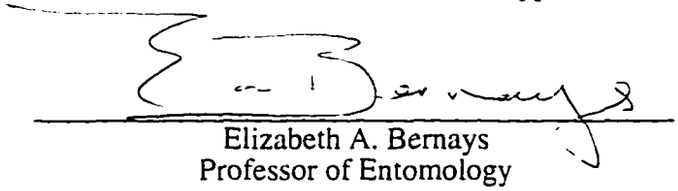

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June 10 1996
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ABSTRACT

The purpose of this thesis is to examine the role that *myo*-inositol plays in feeding and oviposition by the tobacco hornworm, *Manduca sexta.* The results show that; 1) of all of the compounds tested only *myo*-inositol and *epi*-inositol consistently elicited a rapid, phasic-tonic firing response from taste receptors, 2) inositol is a feeding stimulant for both fifth and third stage *M.sexta* larvae, 3) *M.sexta* larvae do not need dietary inositol for normal growth and development, but females need dietary inositol as larvae for egg production as adults, 4) inositol is also a utilizable carbohydrate for *M.sexta* larvae and considering its relative abundance in Solanaceous plants, it may serve as an important carbohydrate source, 5) inositol does not appear to be important in oviposition, 6) inositol is correlated to protein levels in young tomato and tobacco plants.

CHAPTER 1: INTRODUCTION

Chemical stimuli play a major role in the feeding and oviposition behavior of phytophagous insects. These chemical stimuli are important not only in determining if a particular plant is of an acceptable species (Ahmad 1983, Ramaswamy 1988, Hsaio 1985), but may also aid in determining intraspecific host-plant choice (Everly 1959, Jaurnet 1980, Myers 1985, Derridj *et al.* 1989, Papaj and Rausher 1987, Eigenbrode *et al.* 1991).

The sugar alcohol *myo*-inositol is ubiquitous in plants and is found at concentrations that are comparable to some sugars. However, there has been no comprehensive study of the importance of *myo*-inositol to insects. Behavioral and electrophysiological studies show that caterpillars of many species respond to this compound, but there have been few studies aimed at elucidating the significance of this response (Ishikawa *et al.* 1969, Dethier and Kuch 1971, Schoonhoven 1969a and b, van Drongelen and Povel 1980).

The goal of this study is to determine how *myo*-inositol influences the feeding behavior of the tobacco hornworm, *Manduca sexta*, and to relate the behavior to the levels of *myo*-inositol found in their host plants. In addition, oviposition behavior was examined in relation to levels of plant sugars and *myo*-inositol in plant tissues and on the surfaces of foliage.

The following literature review covers contact chemoreception in lepidopteran larvae, caterpillar taste receptors in relation to inositol, the roles that inositol plays in plants, and general information on *M.sexta*.

Contact Chemoreceptors in Lepidopteran Larvae

Caterpillar's taste organs are located on their maxillae and epipharynx (Fig. 1.1). Each maxilla has a palp and a galea. Each galea contains one lateral and one medial styloconic sensillum. Although some work has been done on characterizing the chemoreceptors of the maxillary palp sensilla, most of the work has focused on the styloconic sensilla because they are large. In general each styloconic sensillum is innervated by four chemosensory bipolar neurons for a total of 16 neurons in the four sensilla. Often caterpillars have neurons in the lateral and medial styloconica sensilla that respond preferentially to one class of compound: sugars, inorganic salts, behaviorally deterrent compounds, amino acids, or water (Schoonhoven 1987). In several species of caterpillars, salts appeared to stimulate more than one cell on each styloconic sensillum (Dethier and Kuch 1971, Dethier 1973).

The mechanisms underlying receptor cell sensitivity are mostly unknown. Research in flies for sugars suggest that specific acceptor proteins occur in the dendric membrane. These proteins associate with potential stimulating molecules, having specific molecular configurations, so that only molecules with these configurations are capable of initiating a receptor potential (Hanson 1985).

The sugar sensitive cell in most caterpillar species appears to be moderately specific. It responds to sucrose and to a lesser degree glucose and fructose (Schoonhoven 1973); it appears not to respond to other classes of compounds when they are presented in naturally occuring concentrations. In contrast to this relative specificity, the taste cell for deterrent compounds responds to compounds in several different chemical classes in many species of lepidopterous larvae (Schoonhoven 1974).

A few insect species appear to have taste cells that are specialized for detection of certain host-specific compounds. For example, *Pieris brassicae* have receptors that appear specific for the mustard oil glycosides of their hosts (Schoonhoven 1969b), and

the beetle *Chrysolina brunsvicensis* has a cell that seems to be specialized for hypercin, a compound found in its host-plant, *Hypericum hirsutum* (Rees 1969).

A compound for which some species of caterpillars apparently have a specific receptor cell is inositol, a compound found universally in green plants. Though the degree of specificity is not exactly known, *Bombyx mori* and *Manduca sexta* appear to have taste cells that are highly sensitive to and highly specific for inositol. In *B.mori* this receptor cell is apparently specifically sensitive to *myo*-inositol and *epi*-inositol. Other *neo*- or *rac*-isomers of inositol do not elicit firing of this receptor (Ishikawa *et al.* 1969). This same degree of specificity appears to occur in *M.sexta* larvae also. Schoonhoven (1969b) states "...[in the medial receptor] there is no evidence that any compound other than inositol stimulates the cell. In the case of the lateral inositol receptor, however, some doubt exists in this respect." However, no data is given concerning the actual compounds or the concentrations tested on the *M. sexta* larvae.

Inositol Detection by Lepidopteran Larvae

According to the literature, 30 of the 40 Lepidopteran larvae tested using electrophysiological techniques have the ability to detect inositol with their styloconica sensilla (Table 1.1). In most cases, the literature does not report the specificity of the cell responding to inositol. For all of the species of larvae tested by Schoonhoven, a positive response towards inositol indicates that inositol elicited the greatest response for that one cell. In the lateral sensillum of *D.pini* inositol and glucose fired the same cell (Schoonhoven 1973). Inositol and sugars also appear to act on the same cell in the medial sensilla of *E.acrea, M.americana,* and *I.isabella.*(Dethier and Kuch 1971). But in other cases it was clearly stated that inositol fired a different cell from the sugars. For example inositol fired a different receptor from sugar in the lateral sensillum of *L.dispar* and in the medial sensillum of *I.isabella.*(Dethier and Kuch 1971). However, the only

case in which inositol-specific cells were clearly demonstrated was that of *Bombyx mori* (Ishikawa *et al.* 1969). In this case the receptor cells responded only to *myo-* and *epi-*inositol.

The ability to detect inositol does not appear to be phylogenetically constrained among the species of caterpillars tested. Differences exist between closely related species. In four of the six cases in which two or more species within the same genus were tested, a differences was found. For example, *Pieris rapae* has a taste cell that can detect inositol in the lateral styloconic sensillum, but the congener *P. brassicae* cannot detect inositol. In eight of the ten cases where several species within a family were tested there were variations in the presence and location of the inositol receptor cells. The differences in the presence and location of the inositol receptors along with the differences in sensitivity discussed earlier would seem to indicate considerable evolutionary lability. Figure 1.2 shows the phylogenetic relationship of the superfamilies of Ditrysian Lepidoptera larvae with data on response to inositol mapped on to it.

The ability to detect inositol does appear to be linked to a caterpillar's host-range. When the species of larvae that were tested were grouped into two categories based on the range of acceptable host-plants, polyphagous versus oligophagous/monophagous (Table 1.2), there was a pattern between host-plant range and ability to detect inositol (Chi-square test of independence, $p<0.005$). All of the polyphagous species tested had the ability to detect inositol, whereas only sixty percent of the oligophagous/monophagous species tested could detect inositol. Thus, inositol appears to be more important for polyphagous species than those with a narrower host-range.

Inositol

myo-Inositol is a six-carbon, cyclic sugar alcohol found universally in green plants. Inositol is of considerable importance in plant growth and physiology. It is a key

structural component of phospholipids, and it plays important roles in cell wall formation, osmoregulation, cellular signaling, and phosphate storage. It occurs free in foliage cells at concentrations of several μ mol/g fresh weight.

Structure

myo-Inositol is one of the nine stereoisomeric forms of inositol (Figure 1.3). It is generally regarded as the parent compound among naturally occurring inositols and is undoubtedly the central structure in most aspects of phosphoinositide biochemistry. *myo*-Inositol along with D-*chiro*-, L-*chiro*-, and *scyllo*- and their substituent ethers and esters constitute the major stereoisomers of inositol in plants (Loewus 1990).

Biosynthesis

D-glucose is the precursor of *myo*-inositol. D-glucose is converted to D-glucose-6-phosphate by hexokinase. D-glucose-6-phosphate is then converted to 1L-*myo*-inositol 1-phosphate by L-*myo*-inositol 1-phosphate synthase. The 1L-*myo*-inositol 1-phosphate is then converted to *myo*-inositol by *myo*-inositol monophosphatase. The first committed step in the production of *myo*-inositol occurs with the L-*myo*-inositol 1-phosphate synthase (Billington 1993).

General metabolism of inositol in plants

myo-Inositol plays diverse roles in plants besides its universal appearance as a key structural component of phospholipids. It is an important carbon source for the formation of uronides and pentoses which in turn function as components of cell wall biosynthesis. *myo*-Inositol is also a precursor for sucrosyl oligosaccharides. Methylated forms of inositol such as pinitol and onnonitol appear to play an important role as osmoregulators in several species of plants. Phytate or *myo*-inositol hexa*kis*phosphate is an important

storage form of phosphorous in seeds and pollen grains. Indole-3-acetic acid (IAA) esters of *myo*-inositol and its arabinosyl and galactosyl glycosides constitute an important family of IAA precursors that have an essential role in the homeostasis of this plant hormone. Finally, phosphoinositides and polyphosphoinositides may play a role in signal transduction in plants similar to those found in animal tissues.

Phosphatidylinositol

The biosynthesis of phophatidylinositol is reviewed by Moore (1990). Phosphatidylinositol is a common phospholipidic constituent of cell membranes in plants, exceeded in quantity only by phosphatidylcholine and phosphatidylethanolamine in nonphotosynthetic plant tissues. Phosphatidylinositol and its phosphates may comprise as much as 21% of the phospholipid in a given membrane type and are found in most, if not all, organellar membranes. Photosynthetic tissues contain proportionally less phosphatidylinositol due to the abundance of phosphatidylglycerol in chloroplast membranes. Despite the large body of literature concerning phosphatidylinositol biosynthesis in animals, only a limited number of studies have addressed its synthesis in plant tissue (Moore 1990).

Two reactions for the incorporation of *myo*-inositol into phospholipids in plants have been described. The first involves cytosin diphosphate-diacylglycerol reacting with *myo*-inositol to form phosphatidylinositol in the presence of 3-phosphatidyltransferase. The second is a reaction that simply exchanges *myo*-inositol present in phosphatidylinositol. Polyphosphorylated phosphatidylinositols apparently do not participate in the exchange reaction. Both reactions for phosphatidylinositol synthesis occur in the endoplasmic reticulum of plant cells and possibly the Golgi apparatus (Moore 1990).

Formation of the cell wall components

The importance of *myo*-inositol in the synthesis of cell wall components varies with species and is reviewed by Loewus (1990) and Loewus *et al.* (1990). In one study (Jung *et al.* 1972) using ^{14}C*myo*--inositol-treated *Fraxinus pennsylvanica* callus tissue culture, the label appeared primarily in cell wall polysaccharides (these include the pectic substances) *myo*-inositol glycoside, and phospholipids. In these studies the callus tissue was grown over a 10-week period and the fraction with the highest specific radioactivity was phospholipid, about 300 times greater than the pectic component. A later study by Wolter and Murmanis (1977) which used *myo*-inositol [2-^{3}H]inositol to track deposition of labeled products after 2 weeks of incorporation, found the label almost exclusively limited to the cytoplasmic membranes and virtually none was in the polysaccharidic components of the primary wall. Other studies showed the importance of inositol in production of pectic substances. Helsper *et al.* (1986) tracked ^{3}H*myo*-inositol [uptake by germinating lily pollen. They found lipid-bound tritium appeared shortly after onset of germination but eventually the pectic polysaccharides in the tube wall account for the bulk incorporation of the label (Loewus 1990).

In the proposed pathway for *myo*-inositol oxidation, free *myo*-inositol is converted to D-glucuronate by *myo*-inositol oxygenase and the D-glucuronate is then phosphorylated by glucuronokinase to form D-glucuronate 1-phosphate. Glucuronate-1-phosphate uridylytransferase then converts the compound to uridinediphosphate (UDP)-D-glucuronate. The *myo*-inositol oxidation pathway differs from the nucleotide sugar oxidation pathway in that it is the carbon in the C5 versus the C6 position that is oxidized. Labeling studies have shown that the *myo*-inositol oxidation pathway is quite possibly the primary pathway of carbon from glucose to cell wall uronides and pentoses (Loewus *et al.* 1990).

During cell wall biogenesis, the *myo*-inositol oxidation pathway may have an important regulatory role. UDP-glucuronate and UDP-xylose, especially the latter, are potent inhibitors of UDP-glucose dehydrogenase. Cells that synthesize *myo*-inositol or that are supplied with it from external or internal stores can produce these inhibitors of UDP-glucose dehydrogenase, which will shut down UDP-glucose oxidation and conserve this intermediate for competing processes such as glucan and galactan biosynthesis while carbon furnished through *myo*-inositol continues to flow into pectic polysaccharides (Loewus *et al.* 1990).

Pectic substances produced from inositol are used in wound healing. In one experiment, radiolabeled inositol was injected into the hollow peduncle of wheat plants during the period of caryopsis development. All of the label not translocated into the head remained in the region of the puncture caused by the hypodermic needle and was converted to cell wall pectic substance. The role of *myo*-inositol as substrate for polysaccharide biosynthesis in this type of wounding may be the same as that promoted by insect damage.

Oligosaccharides of D-galacturonate as well as the more complex acidic oligosaccharidic fragments derived from plant cell walls act as signals to activate plant defense mechanisms (Ryan 1987).

Sucrosyl oligosaccharides

Free *myo*-inositol has an important intermediary role in the biosynthesis of oligosaccharides of the raffinose family. Galactinol was biosynthesized from *myo*-inositol and UDP-D-galactose in pea extract. Galactinol functions as a galactosyl donor to sucrose to form raffinose and homologous higher order sucrosyl oligosaccharides. These oligosaccharides are widely distributed in higher plants, where they accumulate in leaves during photosynthesis and in storage tissues such as seed during maturation. Upon

germination these oligosaccharides are converted to cell wall polysaccharides (Loewus *et al.* 1990).

Methyl esters of inositol

Certain species of plants accumulate methyl esters of inositol such as pinitol and quebrachitol. Although the physiological significance of these compounds is not well understood, in some cases they appear to play a role in osmoregulation. For example 70-80% of the monosaccharides in water stressed pine trees was glucose and pinitol. These compounds constituted only 40-60% of monosaccharides in rewatered plants. Since pinitol has such a limited metabolic activity as compared to glucose it would appear that pinitol is important in regulating the osmolarity of the plant cells (Loewus *et al.* 1990). It is also possible that these esters are stores of inositol that can be converted to free *myo*-inositol as determined by metabolic demands (Loewus 1990).

myo-Inositol itself may be actively used as an osmolyte. Salt-stressed tomato plants developed elevated levels of *myo*-inositol when subjected to 100mM NaCl. Resumption of growth in salinated plants was coincident with the attainment and maintenance of elevated *myo*-inositol levels (Sacher and Staples 1985).

Phytate

myo-Inositol is the precursor for *myo*-inositol hexa*kis*phosphate (phytate). Phosphate reserves in seeds, pollen, and vegetative tissues are stored as phytate. Phytate can account for 4-5% of the dry wt of certain seeds and 2% of the dry wt of pollen grains (Loewus 1990).

Indole-3-acetic acid (IAA) ester

Michalczuk *et al.* (1990) reviewed the roles that conjugated IAA esters play in plant metabolism. Indole-3-acetic acid (IAA) esters of *myo*-inositol and its arabinosyl and galactosyl glycosides constitute an important family of IAA precursors that have an essential role in the homeostasis of this plant hormone. Four basic classes of IAA conjugates that have been described of which one is the low-molecular-weight esters such as IAA-*myo*-inositol.

These conjugated forms could act as a kind of "biological zip-code" directing where within the plant the hormone will be delivered. Another possibility is that the molecule to which the hormone is attached could itself be a messenger, directing processes different from those controlled by the hormone. Finally, the conjugation process could be involved in the hormone response mechanism. The attachment of the hormone directly to an internal cell messenger or to a cell protein could function either to activate or to deactivate directly the molecule to which the hormone is conjugated (Michalczuk *et al.* 1990).

Esters of IAA are accumulated in corn kernels during the late stages of ripening and hydrolyzed during germination. These esters often represent more than 99% of the total pool of auxins and may be a storage form of IAA. Furthermore, esterified IAA is the primary if not sole source of auxins for germinating corn seeds (Michalczuk *et al.* 1990). It is possible that IAA is stored in a conjugated form in order to decrease oxidative degradation.

Another role of IAA-*myo*inositol appears to be that of a transport form of IAA. Free IAA from the endosperm of germinating maize seeds was transported only into the mesocotyl, whereas IAA-*myo*-inositol was transported from the endosperm to the shoot at a rate 400 times higher than free IAA. Thus IAA-*myo*inositol appears to supply both free and ester-conjugated IAA to the mesocotyl and coleoptile (Michalczuk *et al.* 1990).

The IAA conjugates appear to be important for hormonal homeostasis appropriate to the environmental conditions. Bandurski *et al.* (1977) showed that etiolated maize seedlings that were given a light exposure sufficient to inhibit the growth rate by 30%, had a 40% decrease of the free IAA concentration and an increase in concentration of esterified IAA.

Second messenger

Coté and Crain (1993) review what is known about inositol trisphosphates as second messengers in plant tissue. In animal tissue inositol 1,4,5-trisphosphate produced in response to environmental signals binds to and opens Ca^{2+} channels in the membrane of the endoplasmic reticulum, allowing Ca^{2+} to flood the cytoplasm, at least locally, and alter cell physiology. A portion of the inositol 1,4,5-trisphosphate produced may be phosphorylated to tetrakisphosphate which in at least some cells may synergistically interact with inositol 1,4,5-trisphosphate to maintain increased Ca^{2+} levels (Coté and Crain 1993).

Researchers assume that a similar system occurs in plants, but have no conclusive evidence yet. In plants the precursors of inositol 1,4,5-trisphosphate, phosphatidylinositol and inositolbisphosphate, are concentrated in the plasma membrane; an observation that is consistent with the presumed role of these lipids in transducing signals from outside to inside the cell.

One of the systems in which inositol 1,4,5-trisphosphate appears to act as a second messenger is in the guard cells regulating stomatal closure. Gilroy *et al.* (1990) showed that inositol 1,4,5-trisphosphate released in guard cells by photolysis of inositol 1,4,5-trisphosphate previously introduced by microinjection produced both a rapid transient increase in cytosolic Ca^{2+} and stomatal closure. Preincubation with extracellular La^{3+}, a Ca^{2+} channel blocker did not inhibit the increase in cytosolic Ca^{2+}

which suggest that the source of the Ca^{2+} is not extracellular. Furthermore, release of Ca^{2+} in guard cells by photolysis of caged Ca^{2+} also produced stomatal closure leading to the conclusion that phosphoinositide metabolism mediates stomatal closure by regulating cytosolic Ca^{2+} levels. Further studies by Blatt supported a model in which Ca^{2+} released by inositol 1,4,5-trisphosphate regulates guard cell turgor by modulating K^+ channel activity (Coté and Crain 1993).

Manduca sexta

Manduca sexta is in the family Sphingidae. The species was originally described by Johansson in his 1763 thesis as *Sphinx sexta* and was reclassified the following year by Linneaus as *Sphinx carolina.* In 1956 Burmeister erected the genus *Protoparce* for the species *rustica.* Since *sexta* is congeneric with *rustica,* it was commonly placed under *Protoparce.* In 1971 Hodges reclassified *sexta* as being in the genus *Manduca.*

Larval *Manduca sexta* feed on plants in the family Solanaceae. As the common name indicates, the tobacco hornworms are a major pest of tobacco. Colonial planters of Virginia and Maryland frequently discussed the damage caused by hornworms in early writings on tobacco farming. Other cultivated plants that are hosts include tomatoes, eggplant, and peppers. In the Tucson Basin area their primary, native host-plants are jimsonweed (*Datura* spp.).

The species is thought to have originated in Southern America and migrated northward through the West Indies to southern Florida and then up through the Southern States, and also north from South America through Central America and Mexico and into the western United States (Madden 1945). The current geographical range of *M. sexta* extends from southern Canada through southern Brazil and thus includes most of the United States, Mexico, the West Indies, Central and Southern America (Madden 1945)

M. sexta overwinters in the ground as pupae, and in most regions the adults eclose in the later summer (Madden 1945). In the Tucson Basin area the moths appear towards the end of the summer monsoon season. The adults are brownish-gray moths that are roughly 4 cm long and have a wing-span of 8 to 12 cm. The species is named for the pairs of orange spots that occur on each of six abdominal segments. The two sexes are similar in appearance. The males are slightly smaller than the females and have larger more complex antennae than the females.

The female moths usually deposit their eggs singly on the undersides of the host leaves (Madden 1945). The eggs are almost round and are 1.25 to 1.5 mm in diameter. The color of the egg can be used to approximately determine its age. The eggs are light green when first deposited and gradually turn to white just before hatching. The larvae generally hatch five days after the eggs are laid at temperatures of 27±2°C (Madden 1945, personal observation)

The newly hatched larvae are about 0.5 cm long and are white to light green. On the eighth abdominal segment the larvae have a dorsocaudal horn, hence the common name hornworm. The larvae become identifiable as the species *M. sexta*, as opposed to other Sphingidae, when they reach the third instar. At this point the larvae are generally green and have seven oblique, black and white stripes on each side of the first seven abdominal segments. The spiracles are bordered in black and the horn usually is red in the later instars. The last instar larvae grow to 7.5 cm to 8.5 cm in length.

Manduca sexta generally have five larval stages. Under ambient summer field conditions in Florida, where temperatures averaged 27±2°C, *M.sexta* were reported to have a larval period lasting from eighteen and a half to twenty-three days 27±2°C (Madden 1945). The larvae used in this study were reared in an environmentally controlled chamber, 27-28:25-26°C, 16h light: 8h dark, on a wheat-germ based artificial diet. Under these rearing conditions the average larval period is approximately 22 days.

Under ideal conditions, the pupal phase lasts 18 days. It is in this stage that these insects diapause, and it is not uncommon for them to remain in the pupal phase for a couple of years (Madden 1945).

In the Southwest USA, *M.sexta* have one and possible two successive generations per year. In warm, moist areas such as Florida and the Carolinas, the insects undergo several successive generations per year.

Table 1.1 Response to inositol by the taste receptors of lepidopteran larvae that have been tested using electrophysiological techniques. + = positive response, - = no response, and * = unclear as to which styloconic sensillum was sensitive to inositol. .

Superfamily	Family	Genus	Species	Medial	Lateral
Noctuoidea	Arctiidae	*Estigmene*	*acrea*[3]	+	-
		Euchaetias	*egle*[2]	-	-
		Isia	*isabella*[3]	+	+
		Pygarctia	*eglenesis*[2]	-	-
	Lymantriidae (Liparidae)	*Euproctis*	*chrysorrhoea*[7]	+	+
		Euproctis	*phaeorrhoea*[6]	+	+
		Leucoma	*salicis*[7]	+	-
		Lymantria	*dispar*[7]	-	-
		Lymantria (Porthetria)	*dispar*[3]	+	+
	Noctuidae	*Catocala*	*nupta*[7]	-	-
		Heliothis	*armigera*[8]	*	*
		Heliothis	*virescens*[8]	*	*
		Heliothis	*zea*[2]	+	-
		Mamestra	*brassicae*[5]	+	+
		Spodoptera	*exempta*[5]	+	-
		Spodoptera	*littoralis*[8]	*	*
	Notodontidae	*Episema*	*caeruleocephala*[7]	+	-
Bombycoidea	Bombycidae	*Bombyx*	*mori*[4]	-	+
	Lasiocampidae	*Dendrolinas*	*pini*[5]	+	-
		Malacosoma	*americana*[3]	+	+
	Saturniidae	*Philosamia*	*cynthia*[6]	+	+
Sphingoidea	Sphingidae	*Celerio*	*euphorbiae*[7]	+	+
		Ceratomia	*catalpae*[3]	+	+
		Laothoe	*populi*[7]	-	-
		Manduca	*sexta*[7]	+	+
		Mimas	*tiliae*[7]	-	-
		Sphinx	*ligustri*[7]	+	+
Papilionoidea	Nymphalidae	*Aglais*	*urticae*[7]	-	-
		Danaus	*plexippus*[3]	-	+
	Papilionidae	*Papilio*	*glaucus*[2]	-	-
		Papilio	*polyxenes*[3]	+	+
		Papilio	*troilus*[2]	+	+
	Pieridae	*Pieris*	*brassicae*[7]	-	-
		Pieris	*rapae*[3]	-	+

Table 1.1 continued

Superfamily	Family	Genus	Species	Medial	Lateral
Hesperioidea	Hesperiidae	*Calpodes*	*ethlius*[3]	-	+
Geometrioidea	Geometridae	*Bupalus*	*piniarius*[5]	-	+
		Operophytera	*brumata*[6,7]	-	+
Pyraloidea	Pyralidae	*Eldana*	*saccharina*[1]	-	-
		Chilo	*partellus*[1]	-	-
		Maruca	*testulalis*[1]	-	-
Tortricoidea	Tortricidae	*Adoxophyes*	*reticulana*[7]	-	+
Cossoidea	Cossidae	*Cossus*	*cossus*[7]	+	-

[1]Den Otter 1992, [2]Dethier 1973, [3]Dethier and Kuch 1974, [4]Ishikawa 1969, [5]Ma 1972, [6]Schoonhoven 1969, [7]Schoonhoven 1972, [8] Blaney and Simmonds 1988.

Table 1.2 Classification of lepidopterous larvae based on their host-range. Species were classified as either monophagous/oligophagous, feeding on one species or species within the same family of host-plants or polyphagous, feeding on several different species of plants.

Species considered monophagous or oligophagous
Those that have the ability to detect inositol:

Leucoma salicis	*Spodoptera exempta*	*Episema caeruleocephala*
Bombyx mori	*Dendrolinas pini*	*Malacosoma americana*
Celerio euphorbiae	*Ceratomia catalpae*	*Manduca sexta*
Danaus plexippus	*Papilio polyxenes*	*Pieris rapae*
Calpodes ethlius	*Bupalus piniarius*	*Adoxophyes riticulana*

Those that cannot detect inositol with their styloconica sensilla:

Euchaetias egle	*Pygarctia eglenesis*	*Laothoe populi*
Mimas tiliae	*Aglais urticae*	*Papilio glaucus*
Pieris brassicae	*Eldana saccharina*	*Chilo partellus*
Maruca testulalis		

Species considered polyphagous
All have the ability to detect inositol:

Estigmene acrea	*Isia isabella,*	*Euproctis chrysorrhoea,*
Euproctis phaeorrhoea	*Lymantria dispar*	*Heliothis virescens*
Heliothis armigera	*Heliothis zea*	*Mamestra brassicae*
Spodoptera litoralis	*Philosamia cynthia*	*Sphinx ligustri*
Papilio troilus	*Operophyhera brumata*	*Cossus cossus*

Fig. 1.1 Diagram of the head of a caterpillar seen from below with enlargements of an antenna and a maxilla. Note the lateral and medial stylocnic sensilla. Each of the sensilla have 4 chemosensory neurons and 1 mechanoreceptor neuron. (Figure from Bernays and Chapman 1994, p. 66)

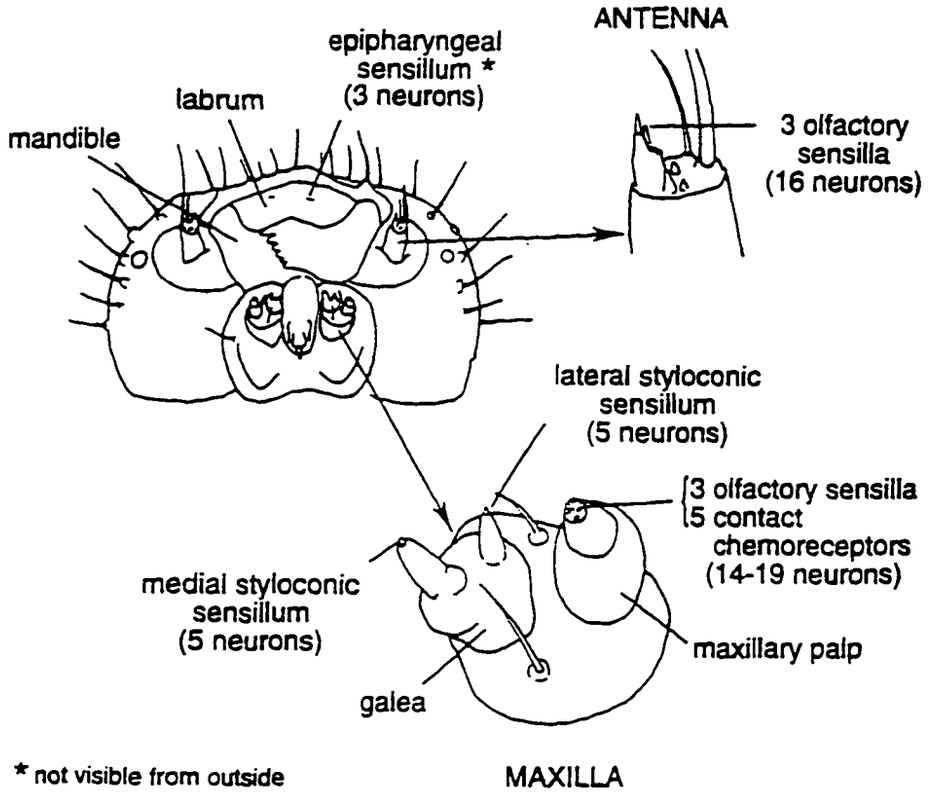


Fig. 1.2 Phylogenetic tree of Ditrysian Lepidoptera. The + and - indicate if members of the families respond/do not respond to when tested using electrophysiological techniques. No sign indicates that no species in that superfamily have been tested using electrophysiological techniques. The * indicates that Yponomeutoidea species have been tested, but not for inositol.

Phylogenetic Tree of Ditrysian Lepidoptera

(Nielsen and Common 1991)

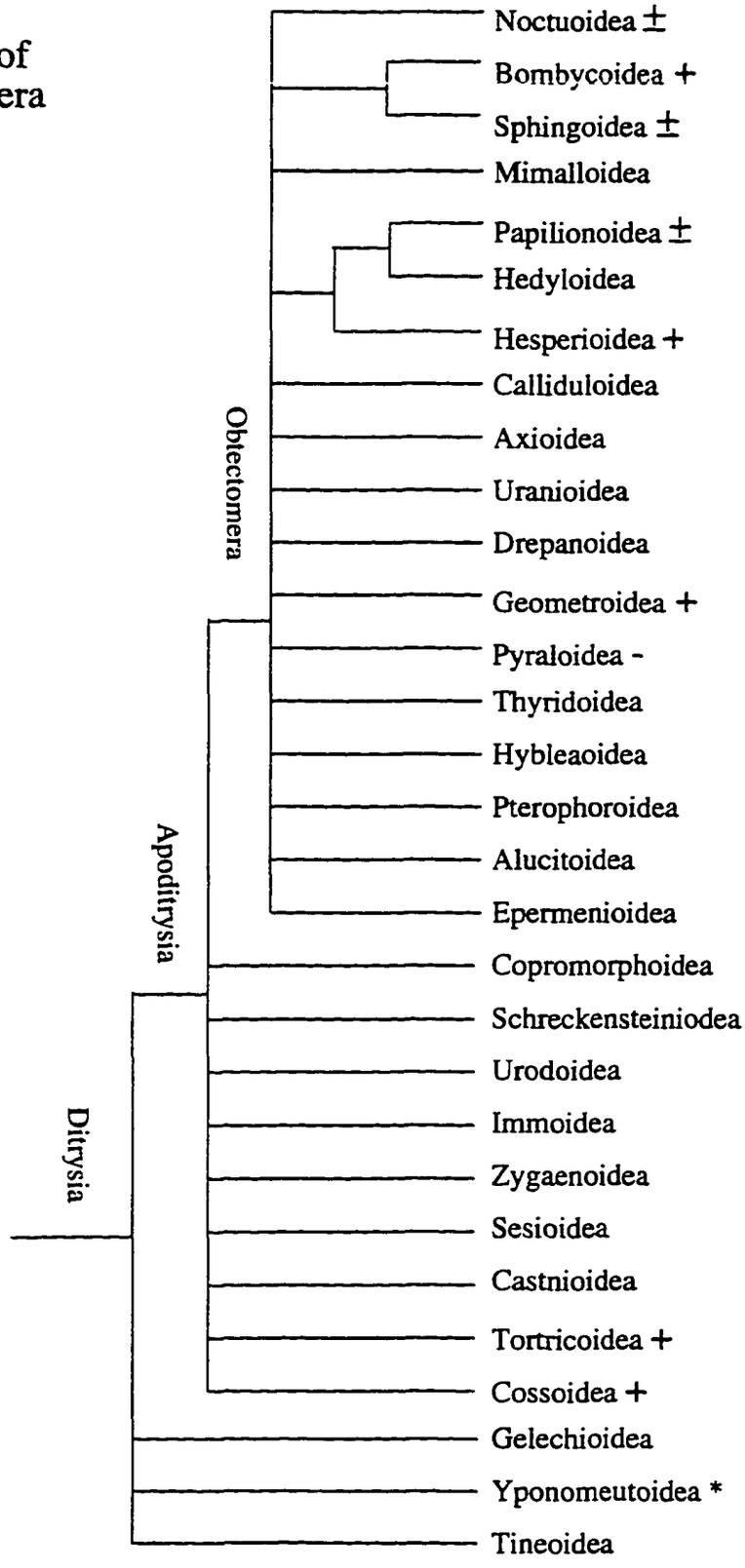
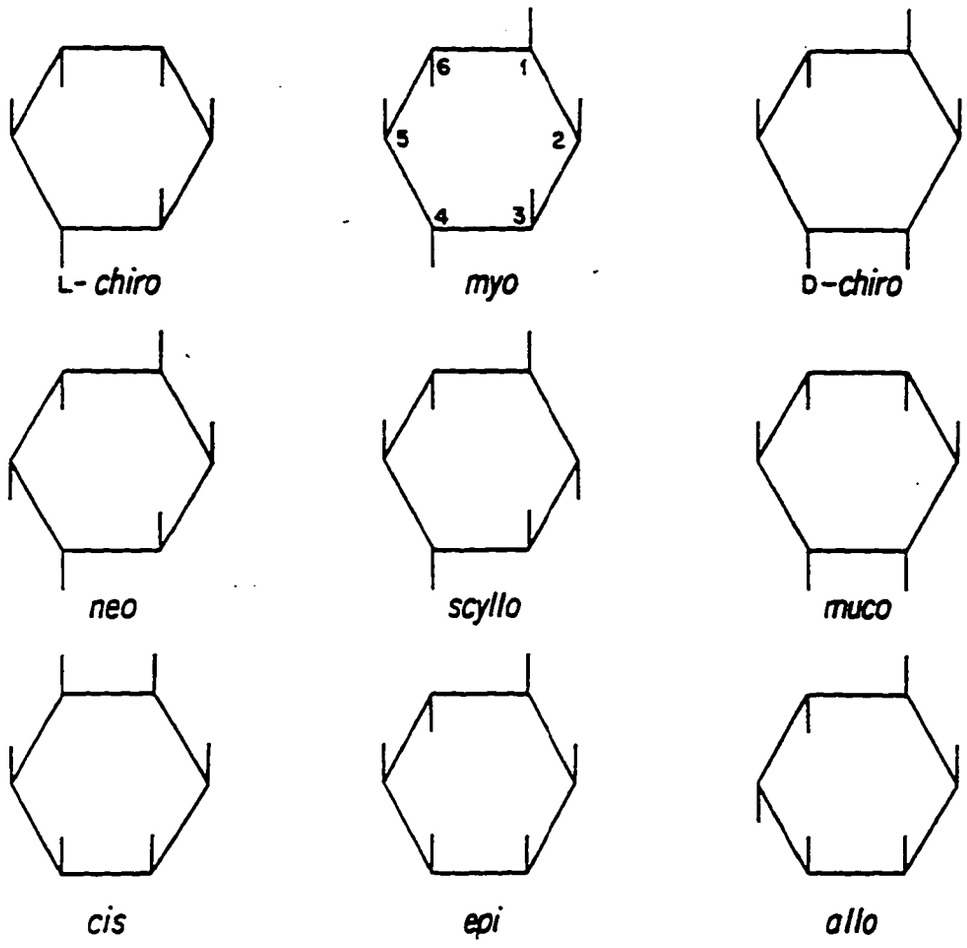


Fig. 1.3 Steriosomeric configurations of inositol (Loewus 1990).



CHAPTER 2: SPECIFICITY OF THE INOSITOL RESPONSE

Introduction

As in most species of caterpillars, the styloconic sensilla of *Manduca sexta* have distinct response patterns to salts, sugars, behaviorally deterrent compounds, and inositol (Fig. 2.1). These response patterns have been generally assumed to reflect the taste spectra of the four sensory neurons found in each sensillum (Schoonhoven 1969a&b, Dethier and Kuch 1971, Dethier 1973, Den Otter 1992). Thus, papers often refer to a salt cell, a sugar cell, a deterrent cell or an inositol cell.

In most insects, however, these sensory neurons are actually only moderately specific. For example in several species of caterpillars, salts appear to stimulate more than one cell on each styloconic sensillum (Dethier and Kuch 1971, Dethier 1973). The sugar cell often responds to several different sugars (Dethier and Kuch 1971, Dethier 1973).

A few species of insects appear to have taste cells that are specialized for detection of certain host-specific chemicals. *Pieris brassicae* larvae have receptors that appear specific for the mustard oil glycosides of their host (Schoonhoven 1969b), and the beetle *Chrysolina brunsvicensis* has a cell that is specialized for hypericin, compound found in its host-plant *Hypericum* spp.(Rees 1969).

In both *M.sexta* and *Bombyx mori*, the inositol receptor is thought to be highly specific. Ishikawa *et al.* (1969) found that the inositol receptor in *B.mori* responded only to *myo-* and *epi*-inositol. Schoonhoven (1969b) stated that the inositol receptor in *M.sexta* was equally specific, but gave no data as to the other compounds tested or the concentrations of the compounds tested.

The specificity of the inositol receptor, if it is truly specific, is surprising. Inositol is ubiquitous in green plants, so unlike the other cases of specific receptors, the inositol receptor is not responding to a host-specific cue. Furthermore, unlike sugars and salts, inositol is not thought to be a major nutrient needed by *M. sexta* larvae.

It is possible that other commonly-occurring plant compounds do stimulate the same receptor as inositol does in *M.sexta*. The apparent specificity of the inositol response may be a result of the limited range of chemicals tested. It is possible that the inositol response is a by-product of the neuron's sensitivity to another plant compound. Since the determination of the exact specificity of the receptor cells is beyond the scope of this thesis, these experiments will focus on the relative specificity and magnitude of electrophysiological responses to selected plant compounds as well as inositol.

The first goal of this chapter is to determine if other commonly occurring compounds found in the host-plants of the *M.sexta* larvae elicit the same type of electrophysiological response as *myo*-inositol. The firing rate and pattern of response was examined for several such compounds to determine if they elicited the same type of response as *myo*-inositol. The second goal is to determine if compounds that are structurally similar to *myo*-inositol elicit the same type of response as *myo*-inositol. Again the firing rates and pattern of responses to these chemicals were compared to those elicited by *myo*-inositol.

Methods

Larvae

M.sexta eggs were obtained from the USDA tobacco hornworm rearing laboratory in Beltsville, MD. The larvae were reared on a wheat-germ based artificial diet under LD 16:8 at 27±3°C at the Arizona Research Laboratory, Division of Neurobiology. All electrophysiological tests were conducted on fifth-instar larvae that were 24-48 hours post-molt.

Electrophysiological recording technique

Electrophysiological recordings were done on both the lateral and medial styloconic sensilla of intact caterpillars using a technique described by Gothilf and Hanson (1994). A latex gasket was placed around the neck of the insects, and the body of the caterpillar

immersed in a 0.1M solution of KCl in a closed glass vial. These conditions induced a state of anesthesia in the larvae. The caterpillar's head was held in place by the latex gasket and its mouthparts were readily accessible to the stimulating/recording electrode. The indifferent electrode consisted of a wire inserted into the bathing medium through the glass vial. This technique was chosen because the caterpillar remains alive and the preparation is viable for several hours longer than the isolated head preparations.

The electrophysiological tip recordings were obtained in the standard manner (Frazier and Hanson 1986). The test compounds were dissolved in a 0.1M KCl solution. A stimulating/recording electrode filled with the stimulating solution was placed over the sensillum. The signals were processed using a high-impedance preamplifier with a baseline-restoring circuit and an amplifier-filter with a band stop set at 130-1200 Hz. Sensory responses to each test solution were recorded over 1 sec. The neural recordings were digitized, analyzed, and stored using SAPID (Smith *et al.* 1990).

All compounds were tested individually at 0.01M concentrations with the exception of glucose which was tested at a 0.1M concentrations. The 0.01M concentration was chosen because when inositol is tested at that concentration it consistently elicits a strong response. It is also a concentration that most closely approximates the naturally occurring concentrations of the first group of compounds to be compared. The higher concentration for glucose was chosen because it is found at concentrations approximately 10X higher than inositol in Solanaceous plants (Chp. 6).

Comparison of responses to myo-inositol and other nutrients found in Solanaceous plants

The first group of compounds tested is present in Solanaceous plant tissues at relatively high concentrations (Fig. 2.2). These included one sugar, three amino acids and five other commonly occurring plant compounds. Since prior investigators have tested the taste receptors' response to sugars (Frazier and Hanson 1986), most of the sugars were not tested in this experiment. Glucose was tested because it was found to consistently elicit a

response in both the medial and lateral styloconic sensilla in previous studies (Glendinning pers. comm.). Glutamine, asparagine, and proline were chosen as representative amino acids because they are known to be found in solution in plant tissue. Rutin is found in concentrations of up to 4% dry weight in some Solanaceous plants and is a phagostimulant for some species of grasshoppers (Bernays, *et al.* 1991). Chlorogenic acid occurs in some solanaceous plants and is a known phagostimulant for some insects (Bernays and Chapman 1994). Ascorbic acid is also an essential nutrient for phytophagous insects and is ubiquitous in plant tissue. Finally, choline chloride was chosen both because it is an essential nutrient and because, like inositol, it is involved in the formation of cellular membranes.

All chemicals tested were examined in comparison with the response to KCl alone (the control solution). This produces an irregular rate of firing with approximately 25 spikes s^{-1} (Fig. 2.1A). The firing rates over the 1s testing intervals were placed into four categories; no increase over the KCl control, a small increase, a moderate increase, or a large increase from the KCl control. Responses that had less than a 50% increase in the total number of spikes generated over the KCl control were categorized as no increase, because there is normally considerable variation in response and up to a 50% increase may be within the range of variation in response to a single stimulus. A small increase was defined as a 50% to a 100% increase in firing rate over the control, and a moderate increase was defined as 100% to 300% increase over the control. Over a 300% increase was counted as a large increase.

The pattern of the responses for each of the electrophsiological tests was also categorized as either no difference from the KCl control, a phasic-tonic response, or a tonic response. Both inositol and sugars generate a phasic-tonic response, which has a rapid but declining firing rate in the phasic portion of the response followed by a steady firing rate in

the tonic portion (Fig. 2.1B&C). Behaviorally deterrent compounds such as caffeine generate a tonic response (Fig. 2.1D).

Comparison of responses to myo-inositol and structurally-related compounds

The second group of compounds tested were structurally similar to inositol, but not necessarily present in Solanaceous plants (Fig. 2.3). These included the sugar alcohols, mannitol and sorbitol, the methylated sugar alcohols, pinitol and quebrachitol, a phosphorylated inositol, inositol-2-monophosphate, and two stereoisomers of *myo*-inositol, *epi*-inositol and *scyllo*-inositol. Inositol-2-monophosphate, *epi*-inositol, and *scyllo*-inositol appear in plant tissues, but only in trace amounts. *epi*-Inositol differs from *myo*-inositol at the sixth carbon and thus has two axial hydroxyl groups, whereas *scyllo*-inositol differs at carbon number two and thus has no axial hydroxyl groups. Each was tested at 0.01M concentrations to compare their stimulating effectiveness with that of *myo*-inositol.

The firing rate and pattern of response elicited by these compounds were compared to that elicited by *myo*-inositol as previously described.

Comparison between the response to glucose and the response to myo-inositol

The responses to glucose and *myo*-inositol were compared using dose-response curves. Glucose was tested at concentrations ranging from 10mM to 250mM. *myo*-Inositol was tested at concentrations ranging from 0.1mM to 50mM.

Comparison between the responses to myo- and epi-inositol

Several different methods were used to further analyze the differences in the responses to *epi*- and *myo*-inositol. First, the total number of spikes generated for each compound over the 1s recording period were counted and the means compared. Second, the temporal patterns of the responses were compared. The temporal patterns were generated by using the instantaneous firing rate for successive 100ms intervals. And finally, the compounds were tested together in a mixture of 0.5mM *myo*-inositol and

0.1mM *epi*-inositol to determine if the firing pattern was indicative of only one dominant cell firing. If the two compounds are firing the same cell then there should be an increase in firing rate over either of the compounds tested singly.

Results

Electrophysiological responses to Solanaceous compounds

Firing rates

In comparison to the other compounds tested, *myo*-Inositol elicited the highest firing rate in most of the larvae tested (Table 2.1). And in all cases, *myo*-inositol elicited a moderate or large response. The response to glucose, the only sugar tested, varied from the small to large category, but were mainly in the moderate category.

The amino acids elicited an increased firing rate in only 54% of the tests, and most responses were small. Two of the four larvae tested responded to glutamine, two responded to asparagine, and three responded to proline. In one of the larvae tested both proline and asparagine elicited a large increase in firing rate. Chlorogenic acid elicited a moderate response in most cases. The response to rutin, ascorbic acid, and choline chloride varied greatly among larvae, but was never large. In general, the change in firing rate to a particular compound was similar in the lateral and medial sensilla.

Firing patterns

The patterns of the electrophysiological responses are shown in Table 2.2. The patterns of responses were not necessarily correlated with firing rate. In some cases in which the firing rate increased, there was no discernible pattern, and in other cases there was a clear pattern, but no increase in firing rate.

Inositol consistently elicited a clear phasic-tonic pattern in both styloconic sensilla. A phasic-tonic pattern was also found with glucose. When the amino acids elicited a response, they produced a tonic firing pattern in the lateral styloconic sensilla and usually a phasic-tonic pattern in the medial styloconic sensilla. In the lateral styloconic sensilla the

pattern of response to rutin was sometimes tonic and sometimes phasic-tonic. However, in the medial sensilla, rutin generally did not elicit a response. In most of the cases, chlorogenic acid elicited a tonic pattern. When ascorbic acid elicited a distinct response it was either tonic or phasic-tonic. Finally, choline chloride generally did not elicit a discernible change in firing pattern.

Comparison of responses to myo-inositol and other structurally related compounds

Table 2.3 shows the firing rates elicited by the compounds that are structurally related to *myo*-inositol. Of these compounds only *epi*-inositol, a stereoisomer of *myo*-inositol, elicited a large response in more than one individual. *scyllo*-Inositol, the other stereoisomer tested, did not elicit a response from the majority of the larvae tested. Most of the responses to the methylated inositols and the other sugar alcohols fell into the small or no response category. The response to sorbitol varied greatly among the individual larvae tested from no response to a strong response. As mentioned in the previous section, the firing rate in response to glucose ranged from a small increase to a large increase

Of the compounds tested only *myo*-inositol, *epi*-inositol, and glucose elicited a phasic-tonic firing pattern on both sensilla in the majority of the larvae tested (Table 2.4). Sorbitol sometimes elicited a phasic-tonic response from the medial styloconic sensilla. Most of the other compounds tested either did not elicit a clear change in firing pattern or elicited a tonic firing pattern.

Comparison between the responses to glucose and the response to myo-inositol

Since glucose elicited a response with a rapid firing rate and a similar firing pattern to *myo*-inositol, further studies were conducted to compare them in more detail. Dose-responses for *myo*-inositol and glucose were compared (Fig. 2.4). These demonstrate that the firing rate in response to *myo*-inositol was much greater than that to glucose at comparable concentrations in both styloconic sensilla. Furthermore, the dose-response curves do not follow the same pattern for *myo*-inositol and glucose.

Comparison between the responses to myo- and epi- inositol

epi-Inositol and *myo*-inositol results were examined more closely to determine whether they were different in either the firing rate or temporal pattern . Examples of the electrophysiological responses are shown in Fig. 2.5. The firing rate for *myo*-inositol was significantly higher than that of *epi*-inositol in the lateral receptor (student t-test, p=0.013) but the firing rates were not significantly different in the medial receptor (Fig. 2.6). The temporal patterns for the responses to *epi*- and *myo*-inositol were similar. *myo*-Inositol eliciting a consistantly higher firing rate throught the time period (Fig. 2.7).

epi-Inositol and *myo*-inositol were also tested together in a mixture. The results of these mixture studies showed only one dominant cell firing (Fig. 2.8 A&B) suggesting that the two compounds fire the same cell. In the lateral styloconic sensilla there was an increase in firing rate for the mixture over either of the inositol isomers tested individually for three of the six insects. In the medial styloconic sensilla by contrast, there was an increase in the firing rate for the mixture over either of the inositol isomers tested individually in all cases.

Discussion

Of the chemicals tested *myo*-inositol elicited the highest firing rate of all of the compounds tested in both the lateral and medial styloconic sensilla. *epi*-Inositol also elicited a rapid firing rate in most of the larvae. Surprisingly, no other plant compounds tested elicited a rapid firing rate in most cases, and the responses to them were highly variable. In some larvae these compounds strongly stimulated a sensillum whereas in others they did not.

The pattern of the response to *myo*-inositol was also consistent among the individuals tested. In all cases *myo*-inositol elicited a clear phasic-tonic firing pattern. *epi*-Inositol also consistently elicited a clear phasic-tonic firing pattern. Furthermore, the patterns elicited by these compounds showed a dominant cell firing which was easily

distinguishable from the salt response. The other compound that consistently elicited a phasic-tonic response was glucose, although the response was less pronounced than that elicited by *myo*- or *epi*-inositol. For the other compounds tested, the firing pattern was extremely variable among the larvae tested. Surprisingly, several compounds including nutrients, elicited a tonic response in many of the larvae. The tonic response is elicited by some behaviorally deterrent compounds such as salicin and caffeine.

Since glucose elicited a rapid firing rate and a phasic-tonic pattern of firing similar to that elicited by *myo*-inositol, further tests were needed to determine if the inositol response was truly different from that of the sugars. The dose-response curves for *myo*-inositol and glucose showed marked differences. The curves did not follow the same pattern and glucose was much less stimulatory than inositol. Thus, it would seem that glucose was firing a different cell than inositol. However, more tests need to be conducted to determine if this is truly the case.

myo-Inositol and *epi*-inositol also elicited similar responses. The firing rate of *epi*-inositol was slightly less than that of *myo*-inositol, but the temporal patterns were extremely similar. When *myo*- and *epi*-inositol were tested together in a mixture, the firing pattern was similar to that which would have occurred if one of the compounds had been tested alone suggesting, but not proving, that the two chemicals fire the same receptor cell. There are two other possible explanations for this result. The chemicals could be firing different cells, and the firing of one cell interferes with the firing of the other cell. Another possibility is that the two chemicals fire different cells that are electrically coupled. Since *epi*-inositol and *myo*-inositol are extremely similar in structure, it would seem likely that the two chemicals are firing the same cell.

Since *scyllo*-inositol, a stereoisomer of *myo*-inositol that lacks any axial hydroxyl groups, did not elicit a response, a high degree of specificity of the inositol response is suggested.

The inositol response seems to be unique in that it consistently elicits a rapid firing rate, has an easily distinguishable firing pattern, and appears to be specific for *myo*- and *epi*-inositol. Since *myo*-inositol is the predominant inositol isomer found in plants, it is probably the only ecologically important stereoisomer of inositol.

Why the larvae have such a strong and unusually consistent electrophysiological response to inositol is still unknown. This unique response suggest that there is something particularly important about inositol. The following chapters will explore the role that inositol plays in feeding behavior, nutrition, and oviposition behavior of *M.sexta*. The levels of inositol found in Solanaceous plants in comparison to the protein and sugar levels will also be examined.

Table 2.1 Percentage of larvae with an increase in firing rate over the salt control for the various plant compounds tested using electrophysiological recording techniques. No increase was defined as less than a 50% increase over the salt control. A 50-100% increase in the number of action potentials generated was classified as a small increase. 100-300% increase in the number of action potentials was a moderate increase. A 300% increase or greater was considered a large increase.

			% of larvae with increase in firing rate over that of the salt control				
Compound Category	**Compound**	**Sensillum**	**None**	**Small**	**Moderate**	**Large**	**N tested**
Inositol	myo-inositol	lat			44	56	9
		med			22	78	9
Sugar	glucose	lat		33	50	17	6
		med		17	67	17	6
Amino acids	glutamine	lat	50	50			4
		med	50	50			4
	asparagine	lat	50	25	25		4
		med	50	25		25	4
	proline	lat	50	50			4
		med	25	25	25	25	4
Other plant compounds	rutin	lat	25	25	50		4
		med	33	33	33		3
	chlorogenic acid	lat	25		75		4
		med			100		4
	ascorbic acid	lat	50	25	25		4
		med	75		25		4
	choline chloride	lat	67	33			3
		med	33	33	33		3

Table 2.2 Percentage of larvae showing either a tonic firing pattern, a phasic-tonic firing pattern, or no discernible firing pattern in response to the chemicals tested.

Compound Category	Compound	Sensillum	% of larvae which had firing patterns			N tested
			No Change	Tonic	Phasic-Tonic	
Inositol	myo-inositol	lat			100	9
		med			100	9
Sugar	glucose	lat		17	83	6
		med			100	6
Amino acids	glutamine	lat	75	25		4
		med	75		25	4
	asparagine	lat	50	50		4
		med	50		50	4
	proline	lat	50	50		4
		med	25	25	50	4
Other plant compounds	rutin	lat		50	50	4
		med	67	33		3
	chlorogenic acid	lat	25	75		4
		med		75	25	4
	ascorbic acid	lat	25	50	25	4
		med	50	50		4
	choline chloride	lat	100			3
		med	67	33		3

Table 2.3 Percentage of larvae with an increase in firing rate over the salt control for the various plant compounds tested using electrophysiological recording techniques. No increase was defined as less than a 50% increase over the salt control. A 50-100% increase in the number of action potentials generated was classified as a small increase. 100-300% increase in the number of action potentials was a moderate increase. A 300% increase or greater was considered a large increase.

Compound Category	Compound	Sensillum	% of larvae with difference in response from that of salt: None	Small	Moderate	Large	N tested
Inositol	*myo*-inositol	lat			44	56	9
isomers		med			22	78	9
	epi-inositol	lat			67	33	6
		med			17	83	6
	scyllo-inositol	lat	67		33		3
		med	67		33		3
Methylated	pinitol	lat	50	17	33		6
inositols		med	71	29			6
	quebrachitol	lat	33	67			3
		med	33	67			3
Phosphorylated	inositol-2-	lat	50	25	25		4
inositol	monophosphate	med	75	25			4
Sugar alcohols	sorbitol	lat	43	43	14		6
		med	14	43	29	14	6
	mannitol	lat	33	50	17		6
		med	67	33			6
Sugar	glucose	lat		33	50	17	6
		med		33	50	17	6

Table 2.4 Percentage of larvae showing either a tonic firing pattern, a phasic-tonic firing pattern, or no discernible firing pattern in response to the chemicals tested.

Compound Category	Compound	Sensillum	% of larvae with the following firing patterns			N tested
			No Change	Tonic	Phasic-tonic	
Inositol	*myo*-inositol	lat			100	9
isomers		med			100	9
	epi-inositol	lat			100	6
		med			100	6
	scyllo-inositol	lat	67	33		3
		med	67	33		3
Methylated	pinitol	lat	100			6
inositols		med	33	33	33	6
	quebrachitol	lat	67	33		3
		med	67		33	3
Phosphorylated	inositol-2-	lat	25	50	25	4
inositol	monophosphate	med	50	50		4
Sugar alcohols	sorbitol	lat	67	17	17	6
		med	33		67	6
	mannitol	lat	83	17		6
		med	50	50		6
Sugar	glucose	lat		17	83	6
		med			100	6

Fig. 2.1 Typical responses to a) salts, b) inositol, c) sugars, and d) behaviorally deterrent compounds by the lateral styloconic sensillum. Figures B & C show a phasic-tonic pattern and Figure D shows a tonic pattern. There is no distinct firing pattern for the salt response.

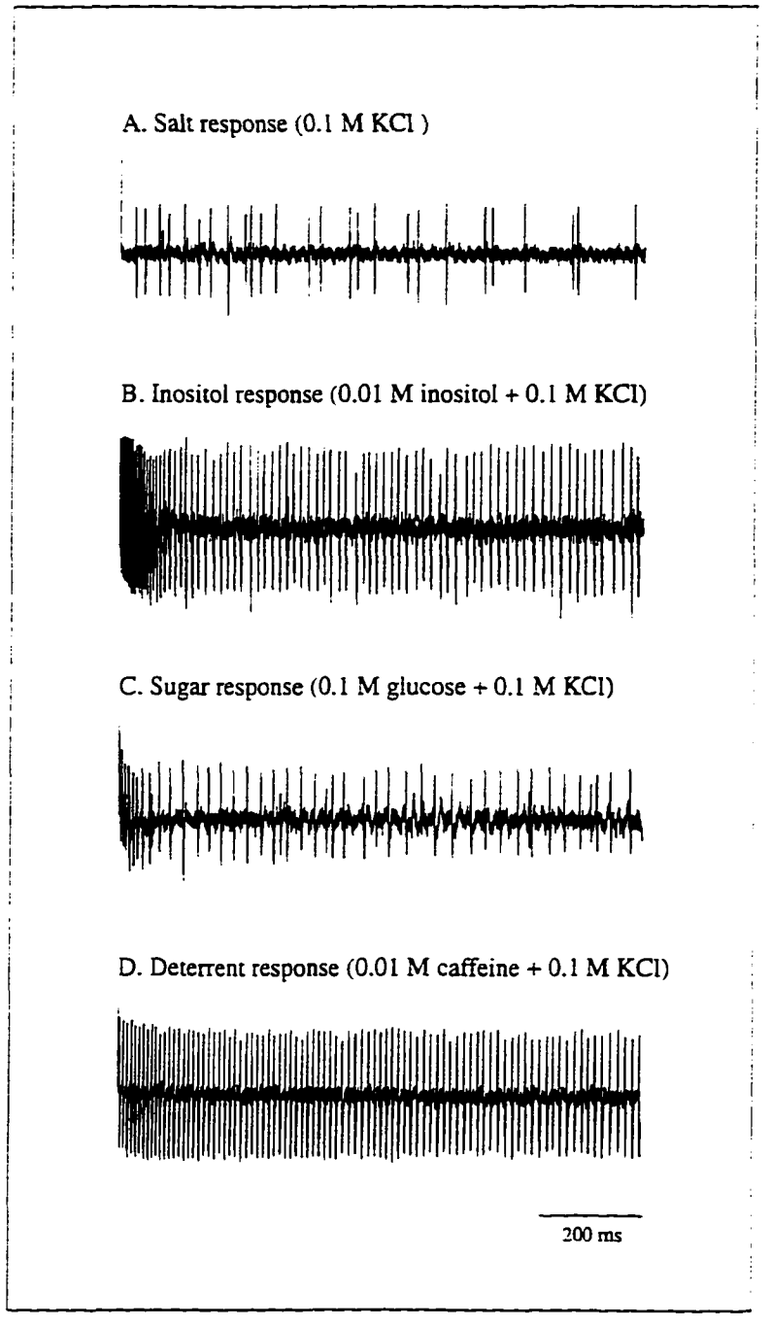
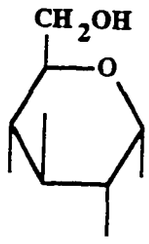

A. Salt response (0.1 M KCl)
B. Inositol response (0.01 M inositol + 0.1 M KCl)
C. Sugar response (0.1 M glucose + 0.1 M KCl)
D. Deterrent response (0.01 M caffeine + 0.1 M KCl)
200 ms

Fig. 2.2 Selected compounds found in Solanaceous plants that were tested on the styloconic sensilla of *M.sexta* larvae using electrophysiological techniques.

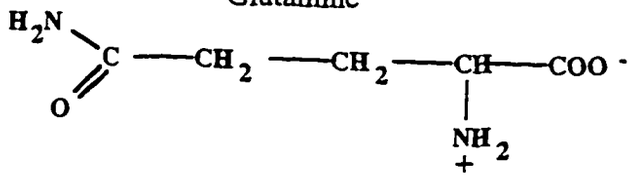
Sugar

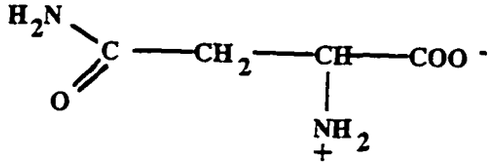


Glucose

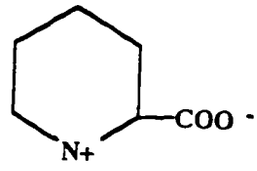
Amino Acids

Glutamine



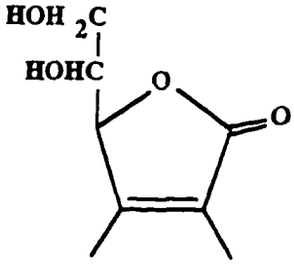


Asparagine

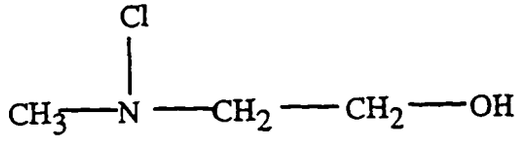


Proline

Other Nutrients

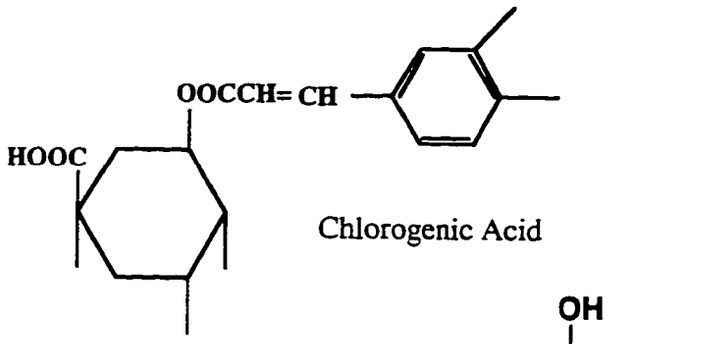


Ascorbic Acid

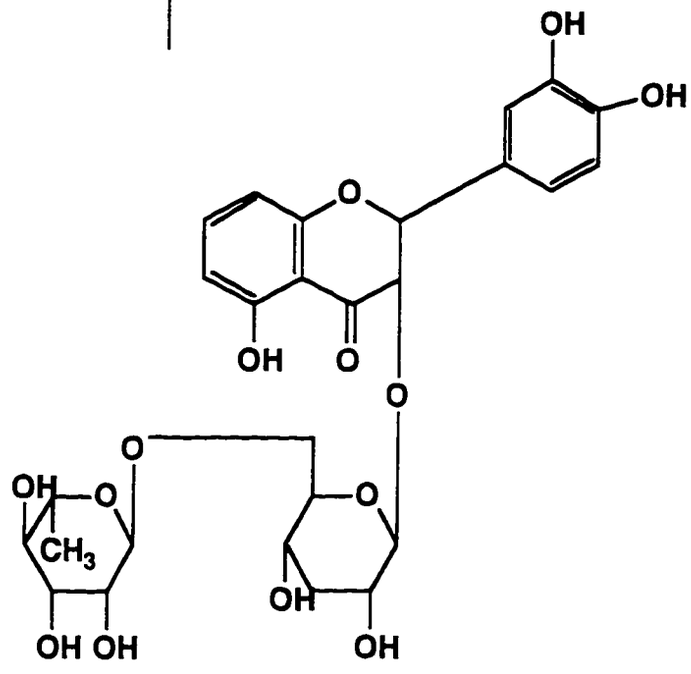


Choline Chloride

Fig. 2.2 Continued



Chlorogenic Acid



Rutin

Fig. 2.3 Compounds similar to *myo*-inositol that were tested on the styloconic sensilla of *M.sexta* larvae using electrophysiological techniques.

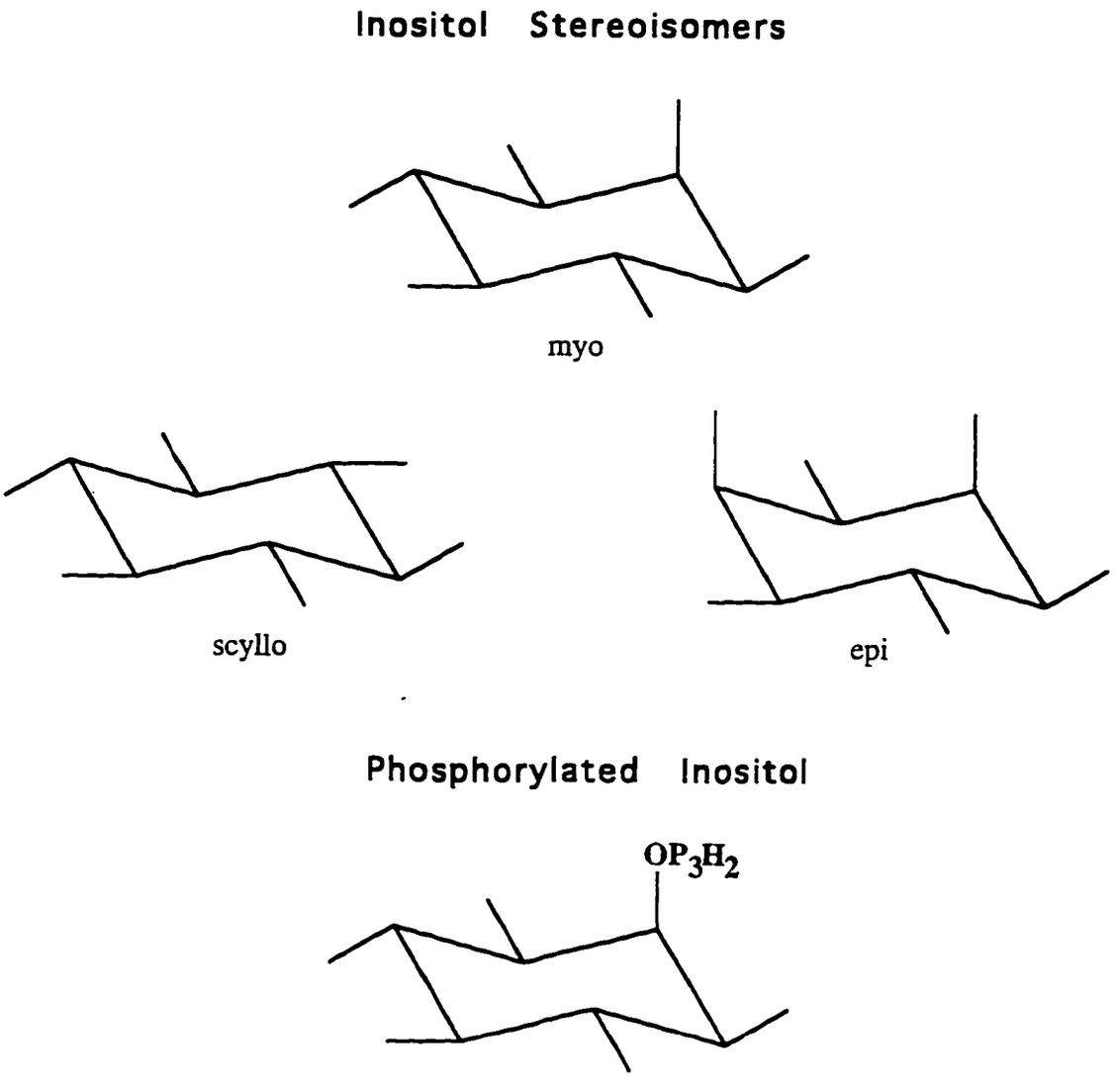


Fig. 2.3 Continued

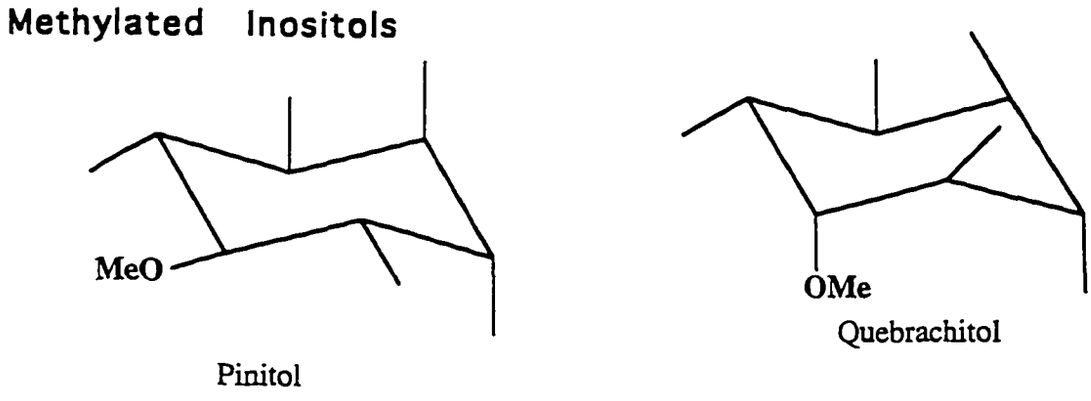


Fig. 2.4 Dose-respnse curves for myo-inositol and glucose for the lateral and medial styloconic sensilla. Ten larvae were tested for each compound. Error bars indicate Standard Error.

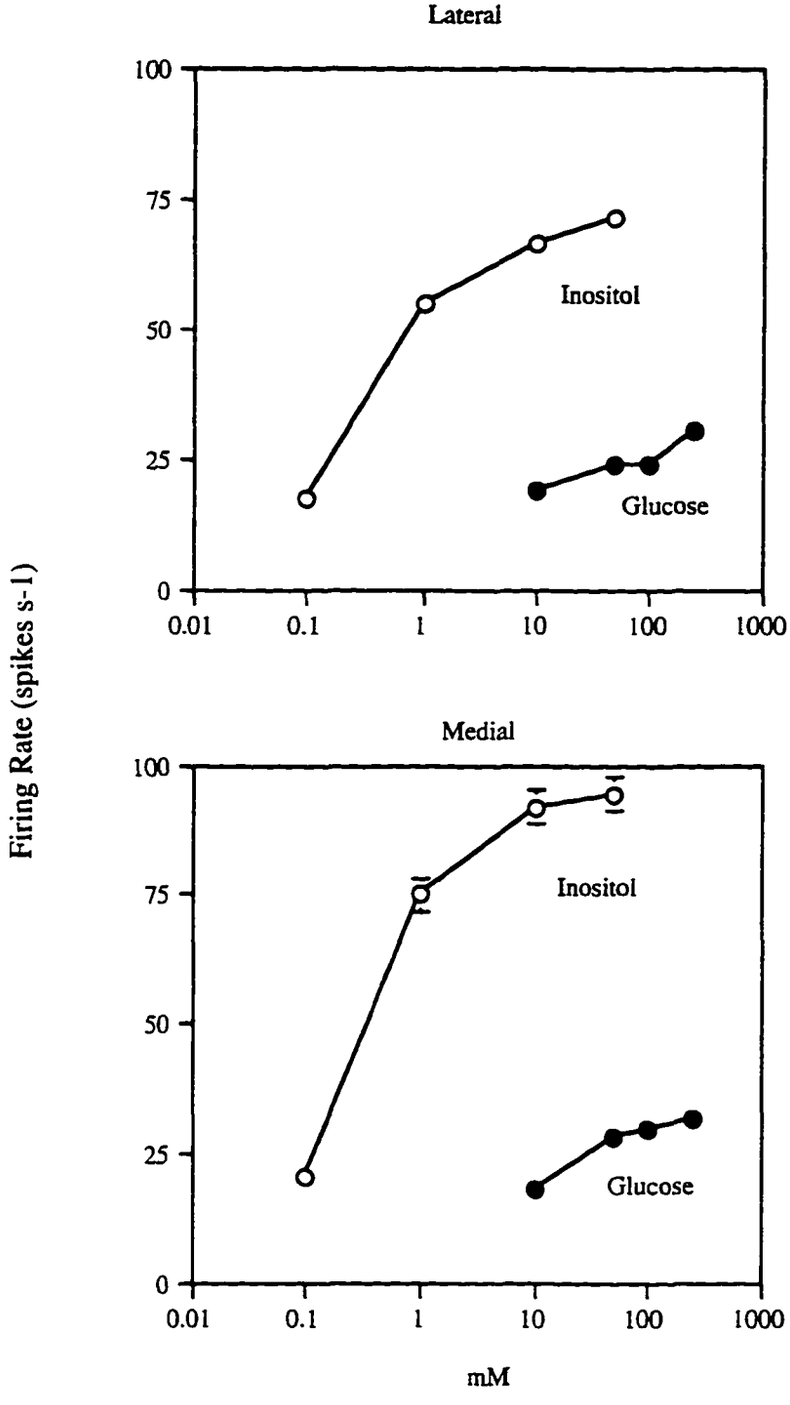


Fig. 2.5 Typical responses by the lateral and medial styloconic sensilla to *epi*-inositol and *myo*-inositol.

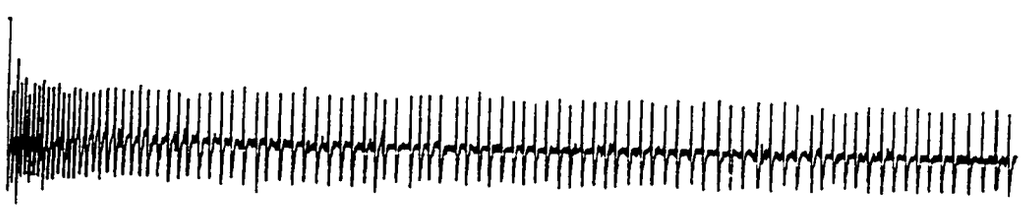
Lateral Styloconic Sensillum

10mM *epi*-Inositol

10mM *myo*-Inositol

Medial Styloconic Sensillum

10mM *epi*-Inositol



10mM *myo*-Inositol

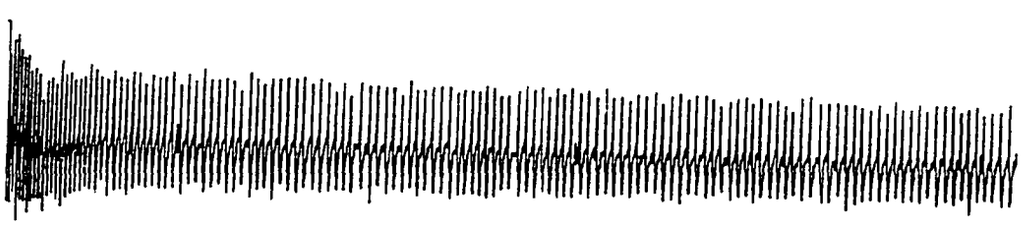


Fig. 2.6 Mean number of spikes fired per second for epi- and myo-inositol in the lateral and medial styloconic sensilla. Each compound was tested on 10 larvae. Error bars indicate S.E. The firing rate for myo-inositol was significantly higher than that of epi-inositol in the lateral receptor (student t-test, p=0.013), but not in the medial receptor.

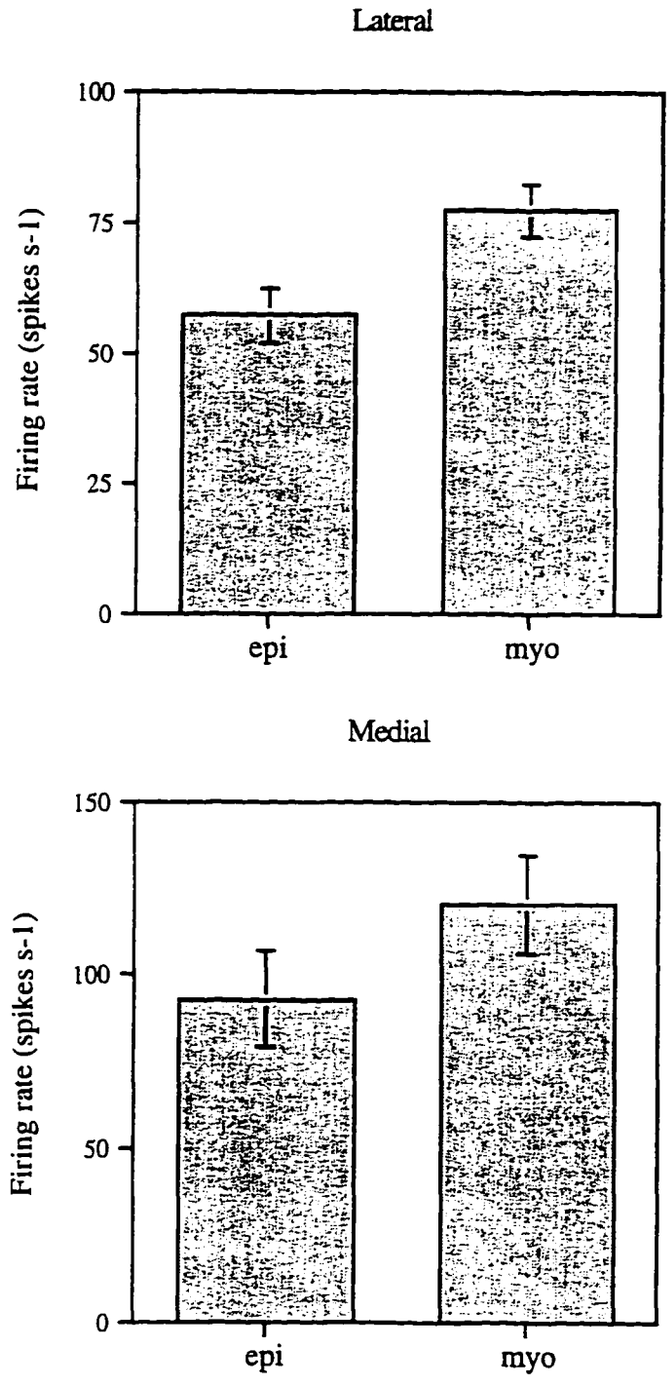


Fig. 2.7 The temporal firing patterns for epi- and myo-inositol. The patterns were generated by using the instantaneous firing rate for each 100ms interval. Error bars indicate standard error. Each compound was tested on 10 larvae.

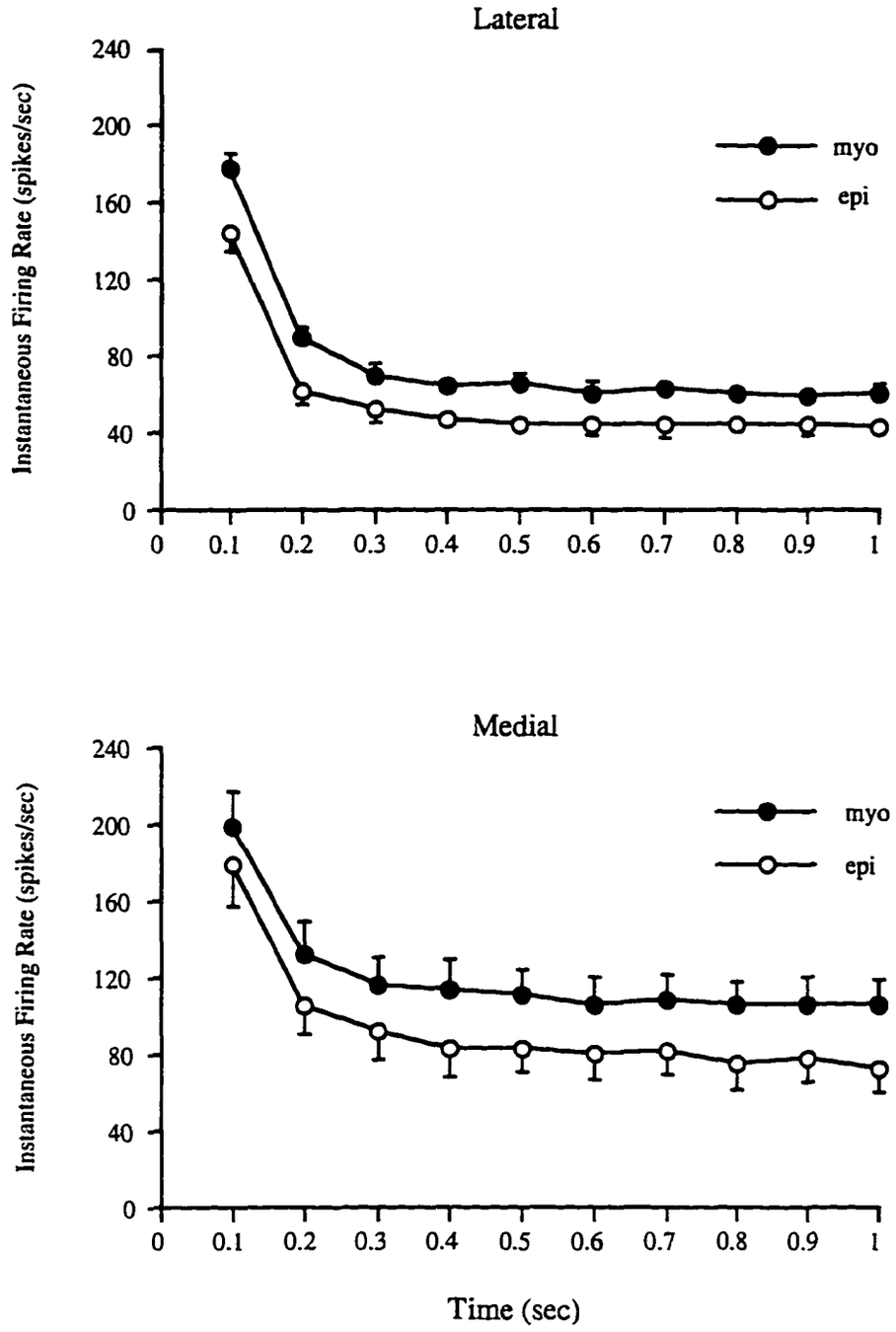


Fig. 2.8 One second traces showing the typical response by the styloconic sensilla to 0.5mM *myo*-inositol, 1mM *epi*-inositol, and a mixture of the two. The response by the lateral styloconic sensilla is shown in A, and the medial response is shown in B.

A. Lateral Styloconica Sensillum Response

0.5mM *myo*-Inositol

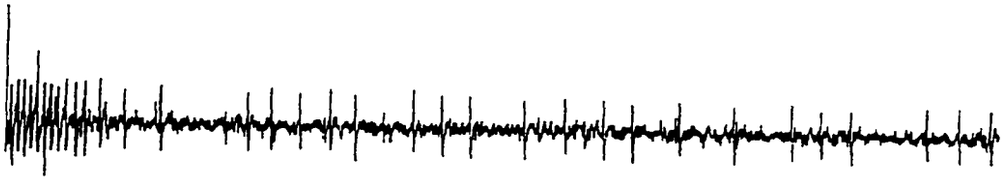
1mM *epi*-Inositol

Mixture of 0.5mM *myo*-Inositol and 1mM *epi*-Inositol

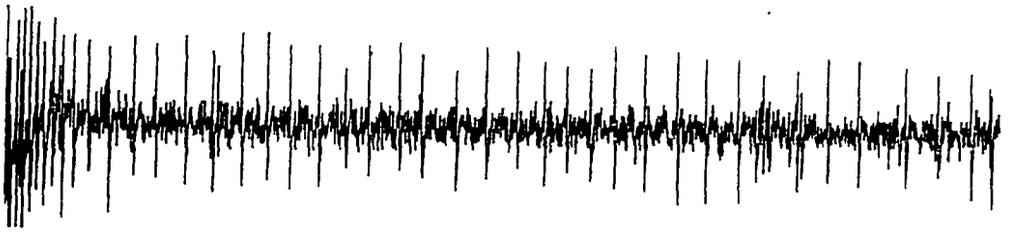
B. Medial Styloconica Sensillum Response

0.5mM *myo*-Inositol

1mM *epi*-Inositol



Mixture of 0.5mM *myo*-Inositol and 1mM *epi*-Inositol



CHAPTER 3: IS INOSITOL A PHAGOSTIMULANT?

Introduction

As discussed in Chapter 1, *Manduca sexta* larvae have receptor cells sensitive to and apparently specialized for detecting inositol (Chapter 2). Inositol was first reported to be a feeding stimulant for fifth instar *M.sexta* larvae by Yamamoto and Fraenkel (1960). They found that for fifth instar larvae, inositol was phagostimulatory when presented alone, and was synergistically phagostimulatory when presented with an unidentified feeding stimulant extracted from Solanaceous plants. Using a two-choice glass fiber disk assay, Städler and Hanson (1978) found that inositol acted as a phagostimulant for fifth instar larvae at 0.01M and 0.1M concentrations. Inositol is a strong enough phagostimulant to make a non-host plant acceptable. Schoonhoven (1969a) found that adding inositol to cabbage made the plant acceptable to the larvae.

The purpose of this study is to further examine how inositol influences *M.sexta* feeding behavior. First, a two-choice glass fiber disk feeding assay was used with fifth instar larvae to determine if: 1) the larvae from the USDA Fargo, North Dakota colony responded to inositol in a similar manner to those from previously reported studies; and 2) to determine if there was a difference in responsiveness between fifth stage larvae that were less than 24 hours post-ecdysis and those that were between 24 and 48 hours post-ecdysis. Second, a no-choice feeding assay was run on both fifth and third stage *M.sexta* larvae. The no-choice feeding assay was used to determine if the presence of inositol influenced feeding behavior by increasing the likelihood that a caterpillar would feed or by increasing the amount that a caterpillar would eat. Finally, since females need dietary inositol in order to produce eggs (Chp. 4), a no-choice feeding assay was used to determine if inositol was more phagostimulatory for females than for males.

Methods

Rearing Procedure

Tobacco hornworm larvae were reared from eggs obtained from the USDA laboratory in Fargo, North Dakota. Approximately 20 eggs were placed in a 6.5 cm diameter clear plastic cup containing a modified version of the artificial diet described by Fernando-Warhakulasuriza (1988) (Table 3.1). The culture was maintained in an environmental chamber on a 16hr light: 8hr dark cycle, 27-28:25-26°C.

When the larvae reached the late second or early third instar, they were transferred to individual cups with fresh diet. Larvae were monitored daily to make sure they had an adequate supply of fresh diet.

Choice Tests

The choice test is a modified version of the two-choice test described by Städler and Hanson (1978). Three test and three control glass fiber discs (GFD, Whatman GF/A, 15mm diameter) were placed in ABABAB order in a circle 25mm from the edge of a Petri dish (15mm high, 150mm diameter). The disks were placed on thumb tacks that were inserted through the bottom of the Petri dish. To keep the disks from touching the bottom of the petri dish, a piece of plastic tubing (5mm high, 3mm diameter) was first inserted over the tack and the GFD placed on top of the tubing. Another piece of plastic tubing was placed on top of the disks to keep them from being pushed off the tacks by the larvae. The disks were moistened completely with either 75µl of a solution of 0.01M *myo*-inositol (test) or with distilled water (control). The test were conducted at a temperature of 28±3°C.

Larval age was carefully controlled in all experiments because previous studies have shown that caterpillar taste receptor responses do not remain constant throughout a larval stadium (Schoonhoven 1976, Simmonds *et al.* 1991). Schoonhoven found that *M.sexta*

taste receptor response towards inositol was maximal between the second and third day of the last larval stage.

Larvae 8 to 24 hours after the molt to final larval instar, day 1, were tested on July 24 and 28, 1993 (n=19 and 16 respectively). On July 13, July 29 and August 1, 1993, day 2 last instar larvae, 24-48 hours post-molt, were tested (n=16, 13, and 14 respectively). A larva was placed in the center of a testing arena and allowed to feed until 50% of the total disks area was consumed (visually estimated). At that point the larva was removed and the amount of inositol disk material consumed was scored visually. Thus, the amount of inositol disk consumed by a larva could range from 0 to 3 disks.

The amount of inositol disk consumed was compared to the amount that would have been consumed based on the null hypothesis, 1.5 disk, using a Wilcoxon signed-rank test. A significant P-value was set at 0.05 for a one-tailed test.

No-Choice tests

In the no-choice assays, each larva was presented with a single glass fiber disk which was moistened with either a solution of *myo*-inositol or distilled water. A test arena was constructed by placing a thumb tack through the center of a plastic dessert cup lid, 6.5cm in diameter. A piece of cork 1cm in diameter and 3-4 mm high was placed on the thumb tack and covered by a piece of scotch tape to keep the solution from penitrating into the cork. A 4.25cm glass fiber disk (Whatman Cat. No. 1820 042) was held on top of the cork with another thumb tack. Each disk was moistened with either 0.6ml of the *myo*-inositol test solution or distilled water. The cups were closed to reduce moisture loss during the experiments. All experiments were conducted at 28±3°C.

M. sexta responses to *myo*-inositol were tested with both fifth and third instar larvae. The fifth instar larval feeding assays were conducted with solutions of 0.1M and 0.01M myo-inositol. All 5th instar larvae were 24-48 hours post molt. The tests dates, treatments, and number of larvae used in each test are shown in Table 3.2. All 5th instar

larvae were fasted 30-45 min. before the beginning of the experiment, and then the larvae were placed in the containers with the disk and allowed to feed for 30 min. The experiments were conducted 3-5 hours after the beginning of the photophase.

Third instar larvae, 24-48 hours post-molt, were tested on disks with either the distilled water control, or 0.001M, 0.005M, 0.01M, or 0.1M *myo*-inositol solutions. The tests dates and number of larvae tested on each treatment are shown in Table 3.3. The third instar larvae were tested 8 hours after the beginning of the photophase. They were not fasted prior to the initiation of the experiment and were left in the assay arenas for 3 hours.

Once the test period ended, the larvae were removed and the amount of disk area eaten was measured either with SigmaScan for the IBM, areas greater than $0.1cm^2$, or visually using graph paper with a $1mm^2$ grid. The area eaten by each larva was recorded. Since the data was not normally distributed, non-parameteric statistical tests were used. To determine if there was an effect of treatment either a Mann-Whitney U tests, for the fifth instar larvae, or a Kruskall-Wallis test, for the third instar larvae, was run on the total data. To determine if the treatment effect was due to larvae being more likely to initiate feeding when presented with a treated disk rather than a control disk, the number of larvae that fed on each treatment was counted and a Chi-square test for the fifth instars, or a G-test for the third instars was run. Finally, to determine if the amount eaten by the larvae that initiated feeding was different when the larvae were presented with control verses inositol treated disk a Mann-Whitney U test, for the fifth instar larvae, or a Kruskal-Wallis test, for the third instar larvae was run.

Sexually-Based Feeding Differences in Relation to Inositol

A set of no-choice feeding assays was run on two day old, fifth instar larvae to determine if feeding response to inositol was influenced by the sex of the insect. Second-day last instar larvae were sexed and weighed. Twenty males and 20 females of similar sizes were placed in individual, no-choice feeding arenas as described above. Each larva

was allowed to feed for 30 minutes on disk moistened with 0.6ml of 0.01M inositol solution. A Mann-Whitney U test was used to determine if there was a sexually-based difference in the overall amount of inositol disk consumed. A G-test was then used to determine if one sex was more likely to initiate feeding on the disks than the other sex. Finally, a Mann-Whitney U test was used to determine if, of the larvae that fed in a treatment, one sex ate significantly more of the disks than the other.

Results

Choice Tests

First-day, last-instar larvae did not show a statistically significant preference for the disk treated with 0.01M inositol solution (Fig. 3.1). However, there was a slight trend for preference for the inositol treated disks. Second-day larvae did prefer the inositol treated disk (Fig. 3.1, $p<0.01$). When the data for the first and second day larvae were combined there was still a preference for the 0.01M inositol treated disks ($p=0.01$, Fig. 3.1).

No-Choice Tests

The results of the no-choice feeding assays show that inositol is stimulatory to both fifth and third instar *M.sexta* larvae. A Mann-Whitney U test showed a treatment effect for fifth instar larvae given the 0.1M inositol disk versus those given the control disk ($p<0.001$, Table 3.4). This effect was due to 1) a higher percentage of larvae feeding on the treated disk versus the controls (Chi-square $p<0.01$, Table 3.5); and 2) of those that fed, the larvae on the treated disk eating significantly more of the disk than the controls (Mann-Whitney U $p<0.05$, Fig. 3.2). A Mann-Whitney U test performed on the data from the fifth instar larvae tested on 0.01M inositol treated disks versus larvae given control disks also showed a significant treatment effect ($p<0.001$, Table 3.6). This effect was due to a significantly greater number of larvae feeding on the treated disks than on the control disks (Chi-square, $p<0.0001$, Table 3.7). Among the larvae that initiated feeding, there

was a trend for the larvae presented with the inositol treated disks to eat more but the results were not significantly different (Fig. 3.3).

A Kruskall-Wallis test on the data from the no-choice feeding assays on the third instar larvae also showed a treatment effect (p=0.01, Table 3.8). A G-test on the number of larvae that fed versus the number of larvae that did not initiate feeding showed a significant treatment effect (Table 3.9). A series of G-tests comparing the number of larvae that fed on the control treatment versus an inositol treatment showed that significantly more larvae fed on the disks treated with 0.01M or 0.1M inositol solutions than the control solution. Although there was no significant difference between the 0.001M or 0.005M inositol solution treatment groups and the control group, there was a trend for more of the larvae to feed if they were presented with treated disks (Table 3.10). A Kruskal-Wallis test comparing the amount eaten by the larvae that initiated feeding did not show a significant treatment effect (Fig. 3.4).

Sexually-Based Feeding Differences in Relation to Inositol

Although there was a trend for females to be more likely to feed on the inositol treated disks than the males, there was no significant difference in either the amount of the disks consumed or the likelihood of feeding between the two sexes. Eleven out of the 20 females initiated feeding, whereas only six of the males consumed any of the disks. The median amount eaten by the females was 0.175 cm^2 . The median amount that the males consumed was 0.00 cm^2.

Discussion

Overall the results of these studies show that inositol acts as a feeding stimulant for *M.sexta* larvae. However, the results from the choice assay were less convincing than those from the no-choice assay. The two-choice assay showed a weak, but significant preference for the inositol treated disks over the control disks for the fifth instar larvae on the second day of the instar, but not the first-day. The results of this study were different than those from a similar study by Städler and Hanson (1978). Städler and Hanson found that inositol was phagostimulatory for recently ecdysed fifth instar larvae at concentrations of 0.01M and 0.1M. The two studies differed in that the larvae in the Städler and Hanson (1978) study were fasted from ecdysis until the time of the test and the tests were terminated when larvae ate 50% of one of the two treatments. By contrast, in the present study larvae were fasted only 30 minutes and the tests were terminated when 50% of the total amount of the disks area was consumed. Perhaps these differences in treatments caused the differences in results.

The results from this study showed increased responsiveness of the second-day, over the first-day larvae, to inositol. These results parallel electrophysiological work done by Schoonhoven (1976) who found that in *M.sexta*, inositol elicited the strongest response by the receptor cells in larvae that were between the second and third day post-ecdysis.

The no-choice feeding assays gave additional information as to the influence of inositol on *M.sexta* feeding behavior. Overall, these assays showed that 0.01M and 0.1M concentrations of inositol were phagostimulatory for both third and fifth instar larvae. However, when the results were broken down, it was found that there were different mechanisms accounting for the treatment effect. For the third instar larvae, the effect of treatment was due solely to an increased probability that the larvae would feed on the inositol treated disks. Once the larvae started feeding, the amount eaten did not differ from that of the control. For the fifth instar larvae, both the probability that the larvae would eat,

and the amount that they would eat, were higher when the larvae were presented with inositol treated disks.

Based on the results from these no-choice tests, inositol appears to stimulate larvae to initiate feeding. In all treatment groups in which the inositol concentration was greater than, or equal to, 0.01M, the larvae presented with inositol were more likely to feed than those presented with control disks. These results suggest that the larvae taste a substrate before feeding and behavioral observations have noted that *M.sexta* larvae palpate a potential food source before biting (pers. obs.).

In nature the presence of inositol on the surface of the plant may influence the larvae to initiate feeding for a number of different reasons. First, the presence of inositol may simply provide a positive cue that indicates plant material. Although inositol is found in all living cells, plants have higher levels of inositol than animals (Loewus 1990). Second, inositol may act as a stimulant for the larvae to initiate feeding because inositol itself is a nutrient for the larvae. Results from Chapter 4 show that the larvae can utilize inositol as a carbohydrate source and that dietary inositol is essential for egg development.

However, tasting the surface of a plant may not give reliable information on the internal contents of the leaf. The results from Chapter 6 showed no correlation between surface sugar levels and tissue sugar levels at least in older plants. These results showed higher levels of inositol in comparison with other sugars on the surface of the leaves than within the tissue.

In assays conducted on third instar larvae, once a larvae initiated feeding on the disk, the amount eaten did not significantly differ between larvae feeding on the treated disk and those feeding on controls. Thus, it appears that inositol acts as a signal to initiate feeding, but does not stimulate the larvae to continue to feed. Generally, increased excitation at the initiation of a meal is thought to increase the amount consumed (Simpson 1990).

There are several possible reasons why inositol is an initial phagostimulant yet its phagostimulatory effects are not sustained enough to increase meal sizes of third instar larvae. One possibility is that the chemoreceptors habituate to inositol within the first few seconds of feeding. Electrophysiological test on the taste receptors show that the receptors adapt to a stimulus and cease to fire within the first seconds of encounter. When the larvae are feeding the taste receptors are in constant contact with the food substrate (Schoonhoven 1969, pers. obs.). Thus, the receptors might habituate during the first few seconds of feeding and no longer relay positive stimuli. However, it is not known if these receptors adapt to the stimuli when the larvae are actually feeding.

Another possibility is that an increase of inositol in the hemolymph may cause decreased sensitivity by the inositol taste receptors. Schoonhoven (1976) found that receptor sensitivity towards inositol may be depressed by adding inositol to the larval diet. Abisgold and Simpson (1988) found that high concentrations of amino acids in grasshopper hemolymph decreased the receptor sensitivity towards amino acids. In this experiment it is possible that as a larva feeds on the treated disks, the inositol concentration in the hemolymph increases which in turn causes the receptor sensitivity to decrease. If these changes in the receptors occur soon after feeding has been initiated, the meal size of the larvae given the treated disks may not be greater than those given the controls.

A third possible reason why inositol did not increase meal size in these experiments is that the ingestion of a carbohydrate only and not proteins or starches may have created an imbalance and a general feeling of "malaise" (Waldbauer and Friedman 1991), and thus the larvae ceased feeding.

Although the presence of inositol did not affect the meal size of third instar larvae, its presence appeared to increase the meal size of fifth instar larvae. The larvae presented with the 0.1M inositol-treated disks ate on average, twice as much as the control larvae. With the 0.01M treatment, there was also a tendency for the larvae to eat more of the treated

disk than the controls. Since dietary inositol is necessary for egg production (Chp. 4), and fifth instar larvae eat most of the food ultimately used for egg production, they may require higher levels of dietary inositol than third instar larvae.

Since dietary inositol is necessary for egg production, it was predicted that female fifth-instar larvae would consume more of the inositol treated disks than the males. Although there was a trend for the females to consume more of the disks, the results were not significantly different. It is possible that further tests would have demonstrated a difference. Also it is possible that male larvae require inositol for successful formation of spermatophores. A study by Turunen (1989) suggests that soluble inositol is transferred from the hemolymph to the testis against a concentration gradient in last instar*Pieris brassicae* larvae.

Although inositol appears to act as a feeding stimulant in these experiments, the larvae only responded to inositol when it was in concentrations five to ten times higher than those that would be encountered in plant tissue (Chp. 6). For reference, 0.001M to 0.005M concentrations of inositol in the glass fiber disk experiment corresponded to naturally occurring inositol levels in tomato and tobacco plants. In both tobacco and tomato plants, the levels of inositol on the surface of the leaves are proportionally higher than those found within the tissue (Chp. 6). At lower concentrations inositol may need to be in a mixture with other compounds in order to be a phagostimulant. For example, Yamamoto and Fraenkel (1960) found that inositol acted synergistically when presented with tomato leaf extracts. Furthermore, electrophysiological assays show that the larvae can detect concentrations of 0.001M inositol.

In conclusion, inositol appears to act as a feeding stimulant for *M.sexta* larvae. This is in general agreement with earlier studies of inositol and *M.sexta* . However, the degree of phagostimulatory effect in this work appears to be considerably less than that found by Yamamo and Fraenkel (1960), Städler and Hanson (1978), and Schoonhoven (1969).

There are several possible reasons for these differences. First, there is a possibility that climatic factors were involved. In Arizona, the very low humidity may have caused insects to often eat the control disks which were treated with water only. This would have had the effect of reducing the apparent stimulatory power of inositol. Second, there are additional methodological differences that may have impacted the results. Previous studies used either dry disks, or inositol in combinations with other plant material. They also were generally 24 hours in length.

The differences in test period may have had particularly important repercussions. In the present work results reflected behavior that occurred for a maximum of two hours, allowing a distinction between initiation of feeding and longer-term intake. Thus, for the third instar larvae, inositol was demonstrated to stimulate the larvae to initiate feeding, but its presence did not appear to increase meal size. In the fifth instar larvae, inositol also acted as a stimulant for larvae to initiate feeding, and only at 0.1M concentration did it significantly increase meal size of the last instar larvae. This is the first set of assays to distinguish between the initiation of feeding and the amount eaten in relation to the presence of inositol. This distinction is important in understanding the role that inositol plays in feeding behavior.

Table 3.1
Diet ingredients for larval *M.sexta* diet.

Vitamin Mix

Niacin(nicotinic acid)	0.5g	Eastman 2266
Calcium pantothenate	0.5g	Sigma P2250 #51H0511
Riboflavin	0.25g	Sigma R4500 #121H0634
Thiamin HCl	0.125g	Sigma T4625 #51H0738
Pyridoxine HCl	0.125g	Sigma P9755 #38F0878
Folic acid	0.125g	Sigma F7876 #40H0321
Biotin	0.01g	Sigma B4501 #120H03035
Vitamin B_{12}	0.001g	Sigma B2876 #85F0549
Distilled Water	100ml	

Dry Ingredient Mix

Casein	125g
Wesson's Salt Mix	40g
Sorbic Acid	10g
Methyl parabin	5g
Ascorbic Acid	20g

Choline Chloride Solution 17.6g/100ml H_2O

Wheat germ 60g

To make the diet boil 375 mls of diH_2O and 7.5 g of agar together in a microwave. Add the wheat germ and mix well with spatula. Add 20 g of dry ingredient mix and mix well. Let cool to 50°C and add 0.5 mls of Vitamin Mix and 0.7mls of Choline Chloride solution. Mix well and pour into dishes.

Table 3.2 Test dates and number of fifth instar larvae tested on either the 0.01M inositol treated GFD, 0.1M inositol treated GFD, or the water treated control disks.

	Dates of Tests			
	Dec. 11, 1993	April 15, 1994	April 26, 1994	Totals
Control	29	12	12	53
0.01M	29	12	11	52
	58	24	23	105

	Dates of Tests		
	Dec. 11, 1993	April 15, 1994	Totals
Control	7	10	17
0.1M	7	10	17
	14	20	34

Table 3.3 Test dates and the number of third instar larvae tested on either the control or inositol treated disks.

	Dates of Test			
	July 31, 1993	Aug. 31, 1993	Sept. 31, 1993	Totals
Control	11	10	9	30
0.001M	12	10	9	31
0.005M	12	10	9	31
0.01M	12	10	9	31
0.1M	12	9	9	30
	59	49	45	153

Table 3.4 Average amount of GFD consumed by fifth instar larvae in a no-choice feeding assay.

	Number of larvae tested	Median	Median Absolute Deviation
Control disk	17	0.4 cm^2	0.4 cm^2
0.1M Inositol disk	17	2.9 cm^2	1.0 cm^2

Table 3.5 Chi-square table comparing the number of larvae that initiated feeding when presented with the 0.1M inositol treated disks to those presented with the control disks.

	# of larvae that fed	# of larvae that did not feed	Totals
Control disks	11	6	17
0.1M Inositol disks	17	0	17
	28	6	34

DF=1, Chi-square=7.29, $p<0.01$

Table 3.6 Amount of GFD consumed by fifth instar larvae on control disks and and disks treated with 0.01M inositol. Larvae ate significantly more of the inositol-treated disks than the controls (Mann-Whitney U tests, $p<0.001$).

	Number of larvae tested	Median	Median Absolute Deviation
Control disk	53	0.0 cm^2	0.0cm^2
0.01M Inositol disk	52	1.25 cm^2	1.25 cm^2

Table 3.7 Table to show the numbers of larvae that initiated feeding when presented with the 0.01M inositol treated disks compared with those presented with the control disks. Inositol caused initiation of feeding.

	# of larvae that fed	# of larvae that did not feed	Totals
0.01M Inositol disks	41	11	52
Control disks	19	34	53
	60	45	105

Chi-square=19.81, DF =1, $p<0.001$

Table 3.8 Amount of GFD consumed by third instar larvae presented with control disks or disks with different concentrations of inositol. Median areas are presented, along with Median Absolute Deviation (MAD). A Kruskal-Wallis test showed a significant treatment effect ($p<0.05$).

	Number of larvae tested	Median	Median Absolute Deviation
Control disk	30	0.0 cm^2	0.0cm^2
0.001M Inositol disk	31	0.03 cm^2	0.03 cm^2
0.005M Inositol disk	31	0.0 cm^2	0.0cm^2
0.01M Inositol disk	31	0.11 cm^2	0.11 cm^2
0.1M Inositol disk	30	0.12 cm^2	0.12 cm^2

Table 3.9 Numbers of third instar larvae that initiated feeding or did not initiate feeding on control disks or disks treated with different concentrations of inositol. Treatment influenced the initiation of feeding.

	# of larvae that fed	# of larvae that did not feed	Totals
Control	9	21	30
0.001M	17	14	31
0.005M	14	17	31
0.01M	23	8	31
0.1M	19	11	30
	82	71	153

DF=4, G-squared = 14.458, $p<0.01$

Table 3.10 Comparison of numbers of larvae that fed on control disks versus number of larvae that fed on each treatment. G-tests were performed for each comparison; P must be <0.0125 to be significant because of the number of comparisons.

Control vs.	G-squared	P-value
0.001M	3.895	0.048
0.005M	1.507	0.220
0.01M	12.361	<0.001
0.1M	6.829	<0.01

Fig. 3.1 Amount of 0.01M inositol disks consumed in choice tests by last-instar larvae. A value of 1.5 indicates that half of the available inositol disks were eaten and half of the control(water) disks were eaten. There was no significat preference for the disks treated with the 0.01M solution of inositol versus the control, water treated disks (Mann-Whitney U test, p=0.35) for the first-day, last instar larvae. However, second-day, last instar larvae did prefer the inositol treated disks ($p<0.01$).When the data were combined there was still a preference for the inositol treated disks($p=0.01$). Graph shows the medians±MAD.

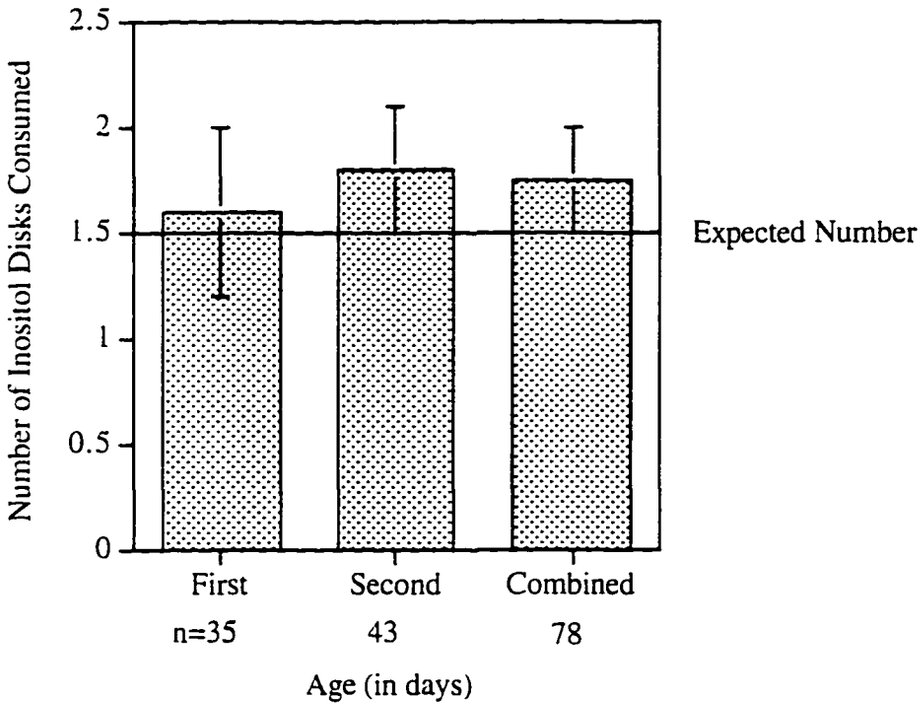


Fig. 3.2 Median amount of 0.1M inositol treated or control disks consumed by fifth instar larvae in a no-choice feeding assay. Only data from larvae that initiated feeding are included. The larvae presented with 0.1M inositol treated disks ate significantly more of the disks than did larvae presented with control disks (Mann-Whitney U tests, $p<0.05$). Error bars indicate MAD.

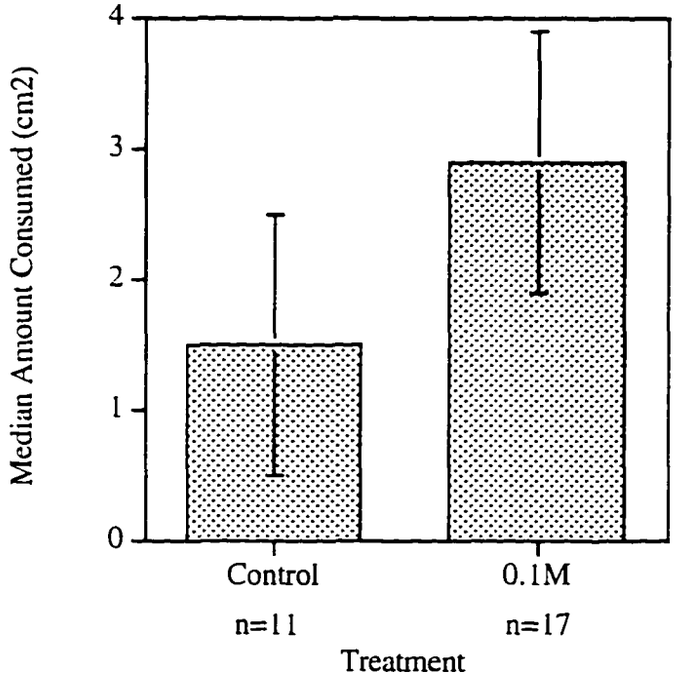


Fig. 3.3 Median amount of 0.01M inositol treated or control disks consumed by fifth instar larvae in a no-choice feeding assay. Only data from larvae that initiated feeding are included. There was no difference in the amount eaten by those presented with the control disks and those presented with a 0.01M inositol treated disks (Mann-Whitney U test, p=0.13). Error bars indicate MAD.

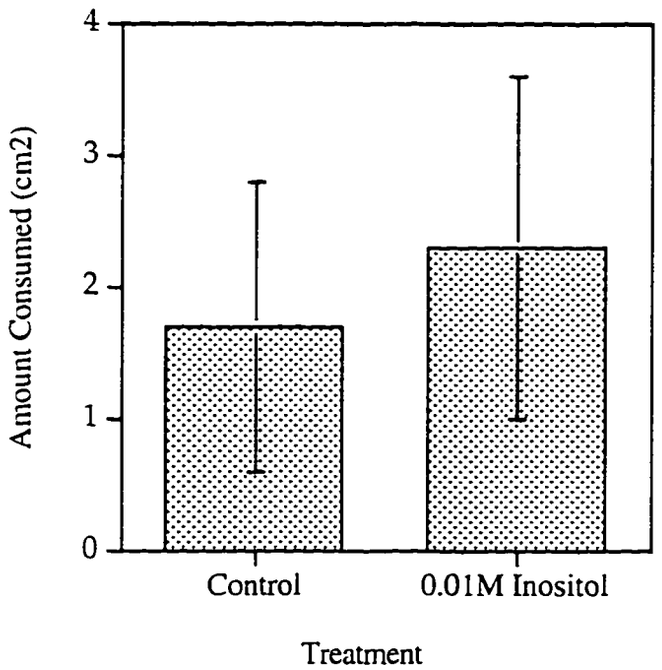
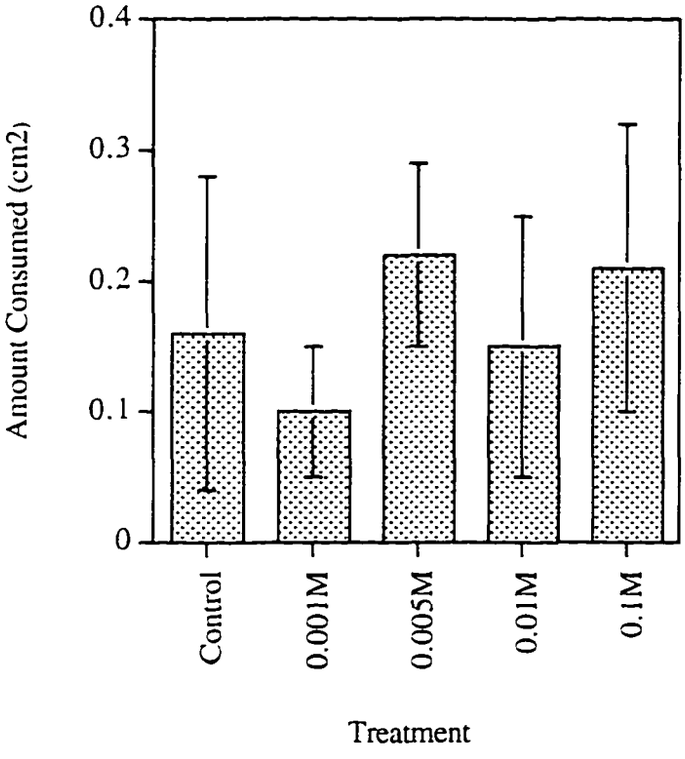


Fig. 3.4 Median amount of disks eaten by third instar larvae in the various treatment groups. Only data from the larvae that fed is included. There was no significant difference in the amount eaten amoung the treatment groups. Error bars indicate MAD.



CHAPTER 4: IS INOSITOL AN IMPORTANT NUTRIENT FOR *MANDUCA SEXTA*?

Introduction

Inositol is an important molecule for living organisms. It is the precursor for phosphatidylinositol which is both a cell membrane component and, in mammalian cells, a precursor for the second messengers diacylglycerol and inositol 1,4,5-trisphosphate. For insects it is known that phosphatidylinositol is also a cell membrane component and it is known that it has the same type of second messenger role in insect cells as in mammalian ones (Berridge 1983).

Although the majority of insects tested can synthesize inositol *de novo* (Dadd 1977), many cannot. Several species of Lepidoptera, Orthoptera, Coleoptera, and Hymenoptera (Dadd 1984) require dietary inositol. Dadd (1961) showed that inositol was necessary for growth, melanization, and pigmentation in locusts. Boll weevils (Vanderzandt 1959), American cockroach (Forgash 1958), and German cockroach (Gordon 1959) required inositol for growth. *Heliothis zea* would pupate, but not emerge if reared on a diet lacking inositol (Vanderzandt 1968).

Bombyx mori caterpillars have taste receptors on their styloconic sensilla that are specific for *myo*-inositol (Ishikawa *et al.* 1969). Studies by Horie *et.al.* (1966) show that *B.mori* require relatively large amounts of inositol in their diets, 1000 µg/g of diet, for normal development. Caterpillars deprived of dietary inositol died within the first couple of instars. Thus, in *B.mori* there is an apparent parallel between the specificity of taste receptors and dietary requirements.

Manduca sexta is also thought to have highly specific taste receptors for *myo*-inositol (Chap. 2). Thus, one goal of this chapter is to determine if *M.sexta* requires dietary inositol.

The second question this chapter addresses is whether or not *M.sexta* larvae are able to use inositol as a carbohydrate source. Chapter 6 shows that the levels of free *myo*-inositol in two species of hosts for *M.sexta*, tomato, *Lycopersicon esculentum*, and tobacco, *Nicotiana tabacum*, were approximately 35% and 14.5%, respectively, of the soluble carbohydrates studied; sucrose, glucose, fructose and inositol . Despite the fact that inositol levels are relatively high in some plants, most insects cannot utilize inositol as a carbohydrate. A review covering 23 species of insects in 6 different orders found that only 3 of the insects could utilize inositol as a major dietary carbohydrate; *Drosophila melanogaster*, *Locusta migratoria*, and *Schistocerca gregaria* (Dadd 1977).

The purpose of these studies is to determine if *myo*-inositol is an essential dietary nutrient for normal growth and development of *M.sexta* and if free *myo*-inositol in the diets of *M.sexta* larvae can be utilized as a source of carbohydrates.

Methods

Preparation of diets

The ingredients for each diet treatment are listed in Tables 4.1-4.3. Instead of using whole wheat germ, which contains inositol, the lipids from the wheat germ were extracted leaving water soluble components, including inositol, behind. The lipid extracts were necessary for growth and development of the larvae. Lipid extracts from wheat germ were prepared by twice extracting the wheat germ for 30min with approximately 200 ml of chloroform each time. The lipid extracts were mixed into the dry ingredients (see Tables 4.1-4.3) and the chloroform was evaporated off at room temperature until there was no chloroform odor present. The dry mixtures were added to the agar and boiling water solutions and mixed well. When the diets cooled to 50°C, the vitamin mix, choline chloride solution, and formaldehyde solution (when used) were added. The diets were stored in sealed containers at 4°C for up to 7 days.

Is inositol a necessary diet component?

In order to determine if *myo*-inositol is a necessary nutrient for normal growth and development of *M. sexta*, larvae were reared on a diet that had no free inositol and their development compared to a control group that was reared on the same diet with added inositol. Development time, pupal weight, and female fecundity were examined. Two experimental trials were run.

The first trial was started on July 5, 1994. The diet components are listed in Table 4.1. The dry mix with the lipid extract added was divided into two equal parts, one for the inositol treatment and one for the control. The diets were then prepared in the standard manner. For each treatment, thirty neonates were placed in individual plastic condiment cups along with a $1cm^3$ block of diet. Larvae were reared under 27:25°C, 16L:8D conditions. The larvae were left in the small containers until they reached the third instar at which point they were transferred to the 6.5cm diameter plastic cups. Larvae were checked daily to make sure that they had an adequate amount of fresh diet.

The second trial was started on March 26, 1995. Approximately 12-24 hours prior to hatching, three eggs were placed in each plastic solo condiment cup along with a $1cm^3$ block of the diet treatment. There were 30 replicates for each of the two treatment groups. The cups were closed and stored in an environmental chamber at 27:25°C, 16L:8D. Within 12 hours of hatching, larvae were removed so that there was only one larvae per cup. No dead larvae were found during this procedure. The larvae were then reared as described in the first trial.

The percentage of insects pupating and eclosing was recorded as well as the time from hatching to pupation and the pupal weights. An unpaired student t-test was used to determine if there were differences in the length of the larval period for different diet treatments. Since female pupae are generally larger than males, the two sexes were separated for the determination of effects of inositol on pupal weight. The pupal weights

were not normally distributed, thus a Mann-Whitney U test was used to determine if there was a diffence in weights due to treatment.

In order to get an estimate of female fecundity, moths were allowed to feed on a 10% sucrose solution for 36-48 hours post eclosion and then dissected to determine the number of eggs greater than 1 mm diameter present for each moth. A Mann-Whitney U-test was used to determine if inositol in the larval diet affected egg production.

Inositol as a carbohydrate source

Three different experimental trials were conducted to determine if *M.sexta* larvae can use inositol as a carbohydrate source. Although the diets and treatment groups differed slightly among the trials, the general protocol remained the same. These trials were started on October 31, 1994; March 26 1995; and April 2, 1995. The diet described in Table 4.3 was used for the trial starting October 31, 1994. The trials starting March 31 and April 2, 1995 used the diet described in Table 4.2.

Approximately 12-24 hours prior to hatching, three eggs were placed in each plastic Solo brand condiment cup along with a $1cm^3$ block of the diet treatment. There were 30 replicates for each treatment group in each trial. The cups were closed and stored in an environmental chamber at 27:25°C, 16L:8D. Within 12 hours of hatching, larvae were removed so that there was only one larva per cup. No larvae died during this procedure. The remaining larvae were given fresh food every other day. Ten days after hatching, the number of larvae surviving in each treatment was recorded and each was weighed.

During the April 1995 experiment, the environmental chamber was accidentally turned off on the fifth day of the experiment. The larvae spent 24h under dark conditions, and were then kept outside the environmental chamber for an additional 24 hours at 25°C while the environmental chamber was re-calibrated. However, all treatment groups within this trial were exposed to the same conditions.

Since the data was not normally distributed, non-parametric tests were used. To determine if there was a treatment effect Kruskal-Wallis tests were run on the data from each trial. The individual treatment groups were then compared within a trial using a series of Mann-Whitney U tests.

Results

Inositol as a necessary nutrient

The length of the larval period did not differ for the larvae reared on the inositol-free diet versus those reared on the diet with inositol (Student t-test, p=0.94, July 1994; and p=0.66, March 1995). In the first trial, July 1994, those raised on the diet with inositol had a larval period of 31.5±.6 (mean±SE) days and those on the inositol-free diet spent 31.6±0.8 days as larvae. In the second trial, March 1995, the larvae on the diet with inositol had a larval period of 27.2±0.5 days, and those on the diet that lacked inositol had a larval period of 26.9±.4 days. For comparison, larvae raised on the normal culture diet pupated 22 days after emerging from the eggs.

Pupal weights were not significantly different for the two treatment groups (Mann-Whitney U tests, Fig 4.1). In both trials males reared on the diet that lacked inositol tended to be a bit smaller than those that were reared on diets containing inositol. However, females reared on the diet that lacked inositol tended to be a bit larger than those reared on the diet that contained inositol.

The percentage of larvae eclosing to adults did not differ with diet treatment. In the first trial 36.7% of the moths eclosed on the diet with inositol and 43.3% eclosed on the diet without inositol. In the second trial 36.7% of the moths eclosed from each treatment group.

The number of eggs greater than 1mm in diameter found in the reproductive system of the dissected female moths was affected by diet (Mann-Whitney U, $p<0.01$, Fig. 4.2).

Females reared on diets that lacked inositol produced a median of 8 eggs, whereas those reared on diets containing inositol produced a median of 45 eggs.

Inositol as a carbohydrate source

The survivorship on the different treatment groups varied among the trials (Fig. 4.3). In the first trial (October 31, 1994), the larvae that were reared on diets lacking a carbohydrate source had a much lower survivorship at ten days than those on diets that contained either sucrose or inositol as a carbohydrate source. In the second and third trials (March 26, 1995 and April 2, 1995), there was little difference among the survivorship rates for the four different treatment groups.

Although larval survivorship at ten days was not effected overall by diet treatment, larval weights were. In all three trials there was a significant treatment effect on larval weights (Kruskal Wallis test, $p<0.001$, $p=0.01$, and $p=0.01$ respectively).

For the first trial (October 31, 1994), a Mann-Whitney U test comparing the treatments showed that the larvae reared on the diet that lacked a carbohydrate source were significantly smaller than those reared on either the inositol or sucrose diet ($p<0.0001$ and $p<0.001$ respectively). There was no difference in weights of the larvae reared on diets that contained inositol and those that contained sucrose ($p=0.37$). The median larval weights $\pm$ the median absolute deviations (MAD) are shown in Figure 4.4.

In the second trial (March 26, 1995), a Mann-Whitney U test comparing the treatment groups showed that the larvae reared on the diet that lacked a carbohydrate source were significantly smaller than those reared on diets that contained either sucrose, inositol, or both sucrose and inositol ($p<0.0001$, $p<0.0001$, and $p=0.0039$, respectively). There was no difference in larval weights among the sucrose, inositol, or sucrose and inositol treatment groups (Fig. 4.5)

In the third trial (April 2, 1995), the patterns were similar to those in the second experiment. The larvae reared on diets lacking carbohydrates were significantly smaller

than those reared on the sucrose diet (Mann-Whitney U test, $p<0.004$). Although the larvae reared on the diets that contained either inositol, or sucrose plus inositol, tended to be larger than those that were reared on diets lacking carbohydrates, the differences in this trial were not significant (Fig. 4.6).

Discussion

The results from this study showed that *M.sexta* larvae do not require free *myo*-inositol in their diets for normal growth or survivorship to adulthood. Larvae reared on diets lacking free inositol did equally as well as those reared on diets that contained inositol, in terms of pupal weight, days to pupation, and percentage eclosing.

These results differed from those found in *B.mori* , another species of Lepidoptera which also has highly specific inositol taste receptors. In *B.mori* the larvae on diets lacking inositol died within the first few instars. Although *B.mori* require dietary inositol, most insects are able to synthesize inositol from glucose (Dadd 1984). Based on the results from this study, it would appear that *M.sexta* are among those insects able to synthesize inositol.

One other possibility is that the *M.sexta* in this study were obtaining inositol from the phosphatidylinositol in the wheat germ lipid extract used in the diets. Larvae would not grow without this extract, even if inositol was included, and thus a completely synthetic diet, free of phosphatidylinositol could not be used. Estimated amounts of inositol possibly present in phosphatidylinositol in the wheat-germ extracts are orders of magnitude less than the amounts that would be required by a species such as *B.mori* . Thus it seems unlikely that this was a major source of inositol.

However, the production of eggs appeared to require more inositol than female *M.sexta* were able to synthesize. There was a significant difference in the number of eggs produced by insects reared on diets containing inositol and those reared on diets that lacked inositol. It is quite possible that several insect species require inositol at least for the production of eggs. For example, although no species of Diptera is known to require

dietary inositol as larvae, adult fruit flies need dietary inositol for egg production (Dadd 1984). *Pimpla turionellae*, a Hymenopterous endoparasitoid, experiences reduced egg production when reared on diets lacking inositol (Ozalp and Emre 1992).

The role that inositol plays in egg production is not known. One possibility is that high levels of inositol are needed to form phosphatidylinositol for cellular membranes.

It is not known if *M.sexta* require inositol for formation of the spermatophore. Work on *Pieris brassicae* strongly suggests that inositol is taken up against a concentration gradient into the testis. However, only 1% of the total amount of labeled inositol was found in the testis. Of the labeled inositol found in the testis, 77% was free inositol and the remainder was phosphatidylinositol. The males may be incorporating inositol into the spermatophores (Turunen 1989).

Results from the second part of this study strongly suggest that inositol is a utilizable carbohydrate for *M. sexta*. In the first two trials, the larvae reared on the diet that contained inositol as the sole carbohydrate source did as well as those reared on the diet that contained sucrose, and both groups did significantly better than those raised on the diet that lacked a carbohydrate. Although in the third trial, insects in the inositol group were not significantly larger than the no-carbohydrate control group, there was no significant difference in larval weights between the groups reared on sucrose or inositol. Overall, the larvae from the third trial were much smaller than those from the other two trials. It was during the third trial that the environmental chamber malfunctioned and thus the larvae were kept at cooler temperatures and under dark conditions for a longer period than in the other two trials.

Results from Chapter 6 suggest that the levels of inositol in both tobacco and tomato leaves are high enough that inositol could be an important carbohydrate source for the larvae. When levels of sucrose, glucose, fructose and inositol were measured in tomato

plants, inositol composed roughly one-third of these compounds on a weight basis. In tobacco inositol made up approximately 15% of these compounds.

Despite the relatively high levels of inositol in some plants and similarity in structure to the sugars, few insect species can utilize inositol as a carbohydrate. Of the 23 insect species tested over a total of 6 orders, only 3 can utilize inositol (Dadd 1977). Dadd (1960) found that inositol supported normal growth in *Schistocerca gregaria* and to a lesser extent it was utilized by *Locusta migratoria. Drosophila melanogaster* is also able to utilize inositol as a carbohydrate source (Dadd 1977) to a limited extent. However, *Bombyx mori*, the only other species of Lepidoptera tested, cannot utilize inositol as a carbohydrate (Dadd 1977).

In mammals inositol is catabolized to form glucose and carbon dioxide in the kidneys (Berdanier 1992). The glucose can then enter the Krebs cycle. Some insects may have a similar system for catabolizing excessive amounts of inositol, although no studies have examined this question.

In conclusion, although free dietary inositol does not appear to be necessary for growth and development of *M.sexta* larvae, it is important for egg production. Inositol also appears to be a utilizable carbohydrate source for *M.sexta* larvae.

Table 4.1 Diet ingredients for the experiment started on July 31, 1994 to determine if *M.sexta* larvae require dietary inositol for normal growth and development. Larvae were reared either on an inositol-free diet or a diet that contained 1% inositol (dry weight).

Dry Mix

Casein	32.5 g
Albumin	10g
Cellulose	20g
Sucrose	20g
Wesson's Salt Mix	4g
Ascorbic Acid	2g
Sorbic Acid	1g
Methyl parabin	0.5g
Lipid Extract from 60g wheat germ	+

Vitamin Mix

Niacin(nicotinic acid)	0.5g
Calcium pantothenate	0.5g
Riboflavin	0.25g
Thiamin HCl	0.125g
Pyridoxine HCl	0.125g
Folic acid	0.125g
Biotin	0.01g
Vitamin B_{12}	0.001g
Distilled Water	100ml

Choline Chloride Solution

Choline Chloride	17.6g
diH_2O	100ml

Diet Ingredients

	Inositol	No Inositol
Dry Mix	33g	33g
Inositol	0.36g	--
Agar	3.75g	3.75g
Water	187.5mls	187.5mls
Vitamin mix	0.5mls	0.5mls
Choline Chloride	0.35mls	0.35mls

Table 4.2 Diet ingredients for the experiments started on Mar. 26, 1995 and Apr. 2, 1995 The treatment groups were a combination of sucrose and inositol, sucrose as the sole carbohydrate, inositol as the sole carbohydrate, and no carbohydrate. Two treatment groups, sucrose and inositol, and sucrose only, from the March experiment were carried on until the *M.sexta* reached adulthood to determine if inositol was an essential dietary nutrient.

Dry Mix

Casein	32.5 g
Albumin	10g
Cellulose	20g
Wesson's Salt Mix	4g
Ascorbic Acid	2g
Sorbic Acid	1g
Methyl parabin	0.5g
Lipid extract from 60g wheat germ	+

Diet Ingredients

	Sucrose & Inositol	Sucrose	Inositol	No Carbohydrate
Dry mix	16.5g	16.5g	16.5g	16.5g
Sucrose	5g	5g	--	--
Inositol	0.25g/0.5g*	--	5g	--
Cellulose	--	--	--	5g
Agar	1.9g	1.9g	1.9g	1.9g
H_2O	94mls	94mls	94mls	94mls
Vitamin Mix*	0.25mls	0.25mls	0.25mls	0.25mls
Choline Chloride*	0.2mls	0.2mls	0.2mls	0.2mls
Formaldehyde	0.1mls	0.1mls	0.1mls	0.1mls

Used 0.25g of inositol in March treatment group and increased to 0.5g in April treatment group.

*See table 4.1.

Table 4.3 Diet ingredients for experiment started on Oct. 31, 1994 do determine if *M.sexta* larvae were able to use inositol as a carbohydrate source. The three treatment groups were sucrose as the sole carbohydrate, inositol as the sole carbohydrate, or no carbohydrate.

Dry Mix

Casein	32.5 g
Albumin	15g
Cellulose	15g
Wesson's Salt Mix	4g
Ascorbic Acid*	2g
Sorbic Acid	1g
Methyl parabin	0.5g
Lipid extract from 100mg of wheat germ	+

Diet Ingredients

	Sucrose	Inositol	No Carbohydrate
Dry mix	24g	24g	24g
Sucrose	5g	--	--
Inositol	--	5g	--
Agar	2.5g	2.5g	2.5g
H_2O	125mls	125mls	125mls
Vitamin Mix	1ml	1ml	1ml
Choline Chloride	1ml	1ml	1ml

*See Table 4.1

Fig. 4.1 Median pupal weights of *M.sexta* reared on diets with inositol and without inositol (Experiments 1 & 2). Error bars indicate Median Absolute Deviation (MAD). There was no significant difference in pupal weights among the treatment groups.

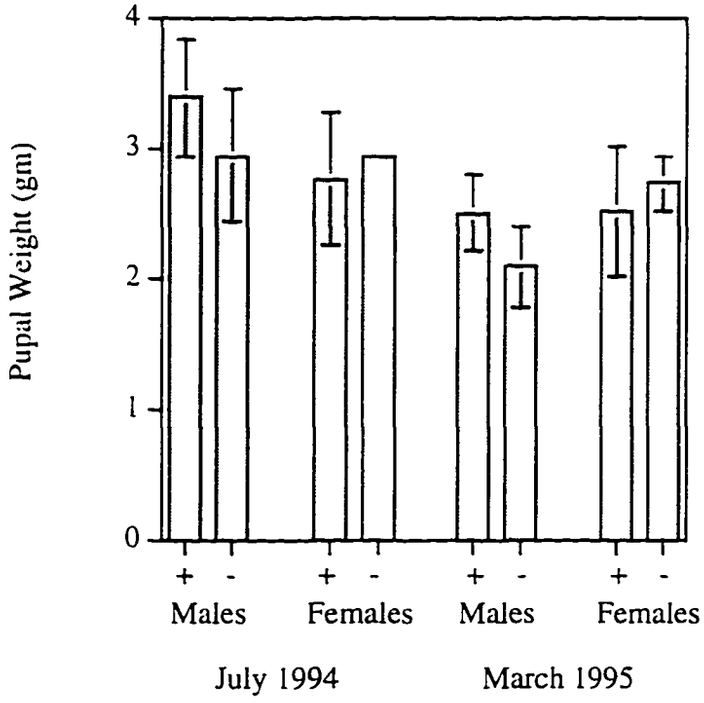


Fig. 4.2 Number of eggs greater than 1cm in diameter produced by moths reared as larvae with and without dietary inositol. The moths were dissected 24 hours post-eclosion and the number of eggs greater than 1mm in diameter was recorded. Insects that were given dietary inositol produced significantly more eggs than those reared on diets lacking inositol (Mann-Whitney U test, $p<0.01$)

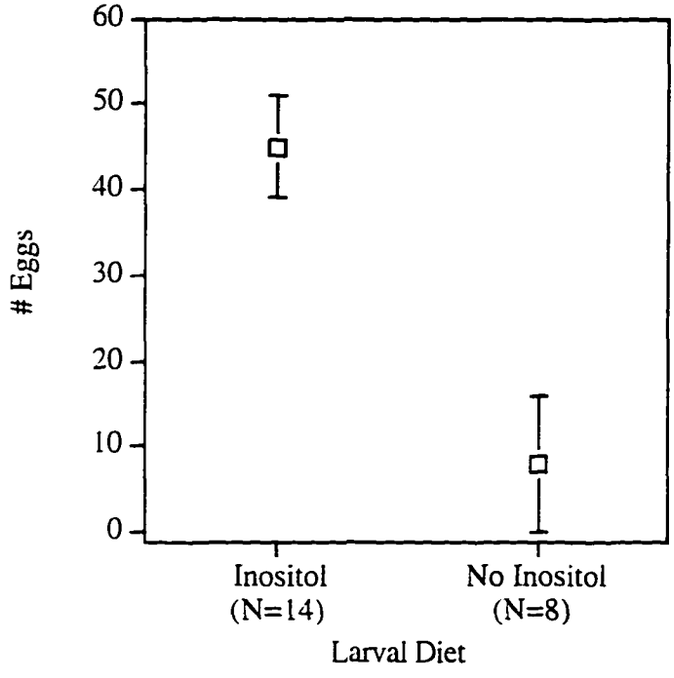


Fig. 4.3 Percent of larvae surviving 10 days after hatching on either diets lacking carbohydrates, diets that contained sucrose as the only carbohydrate source, diets that contained inositol as the only carbohydrate source, or diets that contained sucrose and inositol.

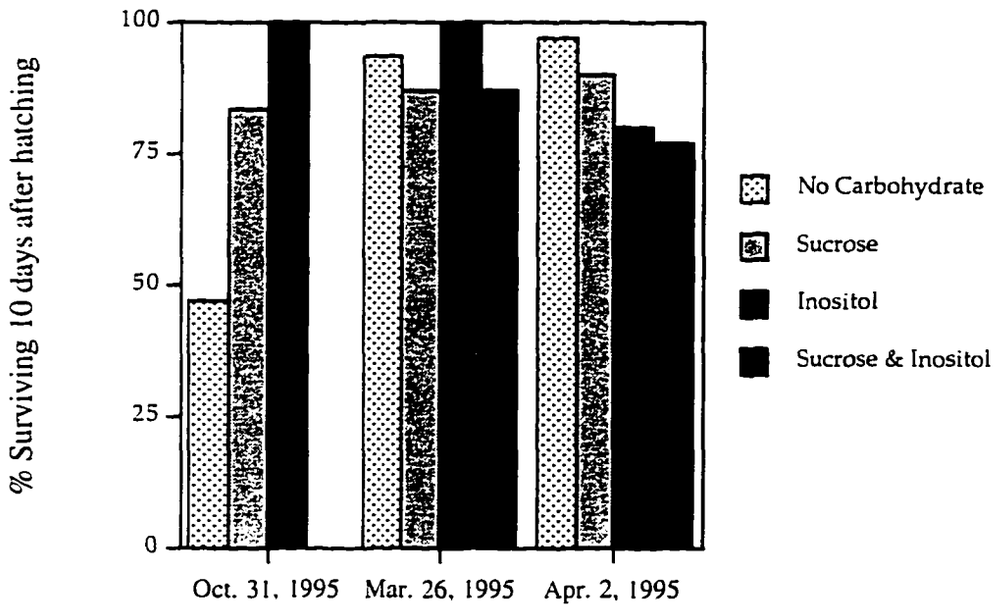


Fig. 4.4 Fresh weights of larvae reared on Oct. 31, 1994 on diets either lacking a carbohydrate source or containing sucrose or inositol as the carbohydrate source 10 days post-hatching. A Kruskal-Wallis test showed a significant treatment effect, $p<0.0001$. A series of Mann-Whitney U tests, in which the p value was set at 0.017, showed that the larvae reared on a carbohydrate-free diet were significantly smaller than those reared on diets containing either sucrose ($p<0.001$) or inositol ($p<0.0001$). There was no difference in larval weights between larvae reared on diets with inositol or with sucrose as the carbohydrate source ($p=0.37$).

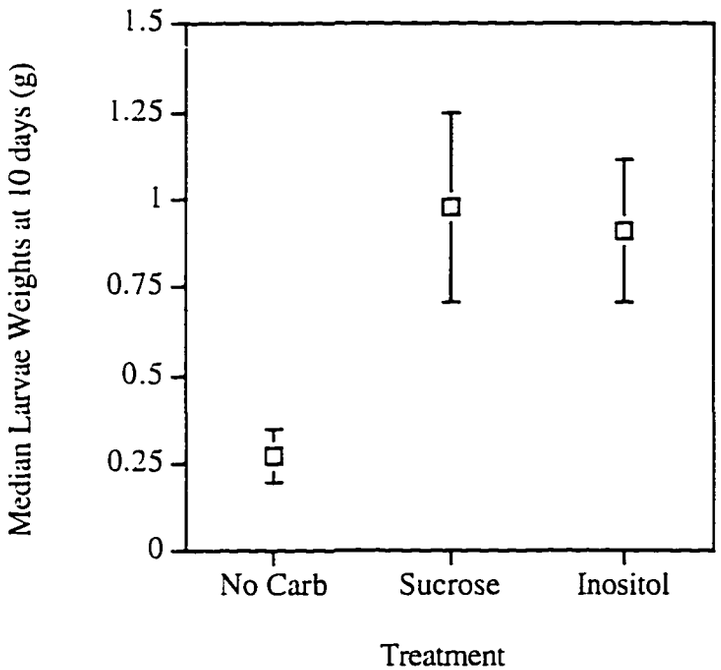


Fig. 4.5 Median fresh weights of larvae reared on March 26, 1995 on diets either lacking a carbohydrate source or containing either sucrose, inositol, or sucrose and inositol. A Kruskal-Wallis test showed a significant treatment effect, $p<0.0001$. A series of Mann-Whitney U tests, in which the p-value was set at 0.01, showed that the larvae reared on a carbohydrate-free diet were significantly smaller than those reared on diets containing either sucrose ($p<0.0001$), inositol ($p<0.0001$) or both sucrose and inositol ($p<0.005$). There was no difference in the larval weights among the larvae reared on the diets that contained either sucrose, inositol, or both sucrose and inositol.

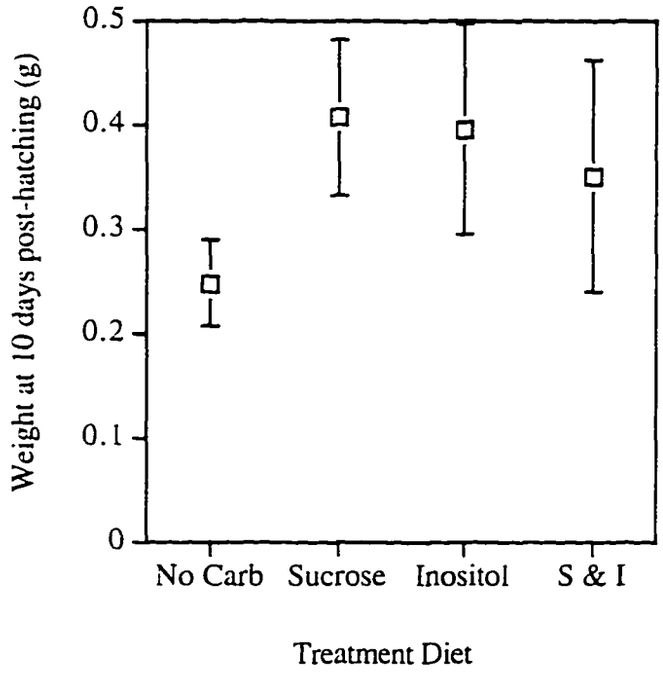
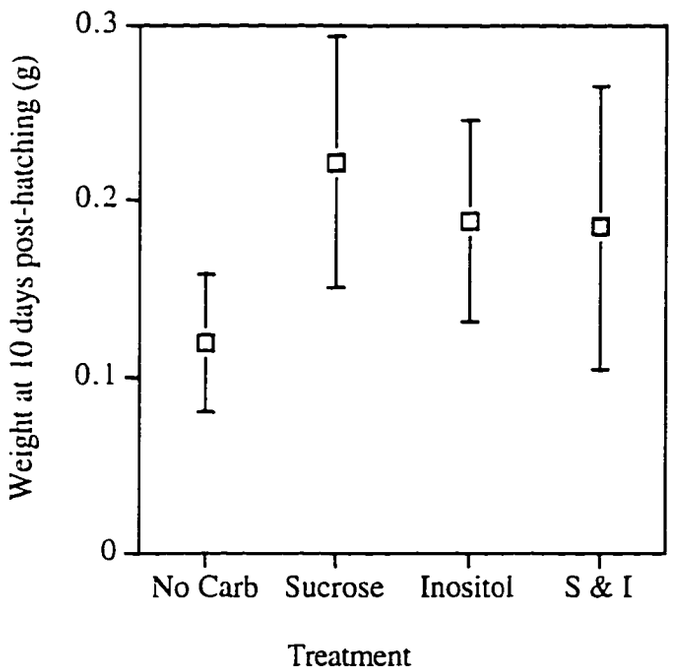


Fig.4.6 Fresh weights of larvae reared on April 2, 1995 on diets either lacking a carbohydrate source or containing sucrose or inositol or sucrose and inositol as the carbohydrate source 10 days post-hatching. A Kruskal-Wallis test showed a significant treatment effect, $p<0.01$. A series of Mann-Whitney U tests, in which the p value was set at 0.01, showed that the larvae reared on a carbohydrate-free diet were significantly smaller than those reared on diets containing sucrose ($p<0.004$), but were not significantly smaller than those reared on the diets that contained either inositol or both sucrose and inositol. There was no difference in larval weights between larvae reared on diets with inositol, sucrose, or inositol and sucrose as the carbohydrate source.



CHAPTER 5: WITHIN-PLANT OVIPOSITION SELECTION BY *MANDUCA SEXTA*

Introduction

The vast majority of research on host-plant selection by ovipositing insects has focused on how insects select the right species of plants. Host selection also involves selecting an individual plant within a species, and within a single plant, selection of parts of the plant that will enable the larvae to feed, develop, and survive. The purpose of this chapter is to examine some of the factors that may influence intraplant selection of oviposition sites by *Manduca sexta* moths.

First instar *M.sexta* larvae have limited mobility on the plants. When 10 neonate larvae were placed on the stem of a tomato plant, all 10 became stuck in the trichome exudents and died within 48 hours (pers. obs.). The trichomes, and exudates from the trichomes, appeared to act as a barrier for movement of the newly hatched larvae. The 6 larvae placed on the leaves of plants began feeding almost immediately regardless of where on the plant the leaves were located (pers. obs.). McFadden (1968) observed that neonate larvae begin feeding almost immediately upon hatching and feed within 1 to 2cm from their egg cases. It was not until the third instar that the larvae are able to move to a new location on the plant (McFadden 1968, pers. obs.). Thus, exactly where eggs are laid is critical in this species.

The nutritional quality of the leaf affects not only the amount of time it takes larvae to develop, but also the chances of surviving. The larvae placed on the younger leaves in the study mentioned above reached the second instar approximately 12 hours before the larvae placed on the leaves from the middle or lower leaves on the plant. Furthermore, larvae on leaves with lower levels of nutrients will need to feed more than larvae on highly

nutritious leaves. Increased time spent feeding has been found to correlate with an increase in risk of predation in some insect species (Bernays in press).

The first questions that this chapter addresses are as follows;

1) are *M.sexta* moths selecting oviposition sites within a host-plant, and if so,

2) do the moths preferentially select leaves on the upper part of the plant?

Previous studies have shown that *M. sexta* prefer to oviposit on leaves from the upper part of tobacco plants (Madden 1945). However, in order to examine possible causes for this choice, we needed to establish that it occurred with our plants and moths. The moths used in this current study have been in culture for hundreds of generations and it was unknown if the extended length of time being reared in culture would affect their ability to select oviposition sites.

The second part of this study examined the details of oviposition behavior of moths in relation to single host plants. The questions addressed were:

1) do the moths preferentially contact leaves on certain parts of the plant more than leaves on other parts of the plant?

2) after contact, do the moths accept leaves on certain parts of the plant more than leaves on other parts of the plant?

The final part of the study addressed whether selection of an oviposition site was in any way related to nutrient carbohydrates and protein. The total amounts of leaflet protein, surface inositol, and surface fructose were measured to determine if their levels correlated with oviposition selection subsequent to the moth making tarsal contact with a leaflet.

Protein was assayed because it is often a limiting nutrient for larval growth (Bernays and Chapman 1994; p.18). Lepidopterous larvae often require dietary protein levels greater than 25% dry weight for optimum growth and development (Scriber and Slansky 1985). The tobacco hornworm, *Manduca sexta*, is certainly no exception. Artificial diets developed for this species contain over 25% protein (Fernando-

Warhakulasuriza 1988). High levels of dietary protein are especially important during the early larval stages (Bernays and Chapman 1994; p. 188). Sub-optimal protein levels cause a decrease in the development rate, adult body size and fecundity (Scriber and Slansky 1981). Thus, one goal of these experiments was to determine if the moths select leaflets with relatively high levels of protein upon which to oviposit.

Inositol, a cyclic sugar alcohol, appears to be important factor for larval feeding; it is a phagostimulant for *M.sexta* larvae. Since inositol is found in all plants, the larvae cannot be using it as a host-specific cue. However, it is possible that they are using this chemical to determine overall host-plant quality or some particular factor, such as protein levels. This would be adaptive if there is a correlation between levels of inositol and protein. Since inositol is a phagostimulant for the larvae, it is hypothesized that the moths would select leaflets with high levels of surface inositol.

Finally, fructose was assayed because studies on the European corn borer showed that the levels of fructose on the plant surfaces were highly correlated with insect oviposition preference (Derridj *et al.* 1989).

Methods

The moths used in these experiments were reared from eggs obtained from the USDA rearing facilities in Fargo, ND. The larvae were reared as described in Chapter 3. Pupae were sexed and sorted according to sex three to four days following pupation. The pupae were placed in clear, 8L plastic buckets with wire screened lids. The bottoms of the buckets were lined with paper towels, and paper towels hung down from the top of the bucket so that the newly emerged moths would be able expand their wings. The buckets with the pupae were stored in a screenhouse on the University of Arizona campus.

All experiments were conducted in a large screenhouse and thus were conducted under ambient summer conditions. Male and female moths were placed in the cages the evening after they eclosed and allowed to pair for at least one night before the experiment

began. On these nights tomato and tobacco plants were placed just outside the cages so that the moths could smell, but not touch, the plants. Cotton wicks soaked in sugar water solutions were placed in the cages for the moths to feed upon.

Egg-distribution

The first series of oviposition experiments was conducted to determine if the population of *M.sexta*, which had been in culture for many generations, still exhibited oviposition selection behavior between and within plants. Manduca deposits single eggs and during a bout of egg-laying an individual moth makes multiple visits to a plant in the screenhouse. The moth touches leaf after leaf, ovipositing on some occasions and not others. If oviposition choice were completely random, then the distribution of eggs across leaves on a plant should follow a Poisson distribution. So, a first approach to examination of choice behavior was to compare actual distribution with a Poisson distribution.

The first experiments to study egg-distribution patterns were conducted on July 16 and 17, 1993. Three tobacco plants (*Nicotiana tobacum*), two tomato plants (*Lycopersicon esculentum*, Better Boy), and two basil plants (*Ocimum basilicum*) were placed in a 2mX2mX2m field cage. All plants were in one gallon pots. The tobacco plants had two to three stalks per plant, and each plant had 18-28 leaves. The tomato plants had six or seven fully expanded leaves. The plants were placed in the cage in the mid-afternoon and were removed late the following morning. The egg distributions for each plant and leaf were recorded and the eggs were removed. The plants were returned to the cages and the experiment repeated with the same plants and moths. On July 16, five female moths were present. However, only three female moths were present on July 17.

In a second experiment to further examine within plant selection, two small cages 0.5mX0.5mX0.5m were set-up on July 16, 1993 each with a single female and a single tomato plant. In the first cage the tomato plant had six fully expanded leaves and in the

second cage the tomato plant had seven fully expanded leaves. The egg distribution by leaflet was recorded for each plant.

Results were analyzed by comparing the actual distributions by leaf for tobacco plants, or leaflet for tomato plants, to the calculated random, or Poisson distributions, using Chi-square goodness-of-fit tests.

To determine if more eggs were laid on leaves on the upper part of the plant, the plants were visually divided into upper and lower halves based on leaf number. If an odd number of leaves was present, the extra leaf was assigned to the lower half of the plant. Since lower leaves also tended to be larger, the lower half of a plant generally had greater leaf surface area. This means that strictly more eggs would be expected on the bottom half if there is no oviposition bias. However, the expectation was that more eggs would be laid on the upper half, so that these tests were very conservative.

The number of eggs present on the upper and lower leaves was counted and these numbers were compared to the expected values using a Chi-square goodness-of-fit test. The expected number of eggs in each plant half was determined by taking the total number of eggs present and dividing by two.

Oviposition in relation to leaflet position, protein level, surface fructose level, and surface inositol level

Observation experiments were conducted in the summer of 1994 in order to answer the following questions:

1) do *M.sexta* select for leaflets to oviposit upon prior to landing on the leaflets?

2) are leaflets with high protein levels preferred?

3) is the surface fructose level correlated with ovipositon?

4) is the surface inositol level correlated with oviposition?

All of the behavioral assays were conducted in a 2mX2mX2m field cage which was set-up inside a large screenhouse. Thus, the experiments were conducted under ambient

summer conditions during the hours of 9pm to 1am. Red lights were used in order for the investigators to observe the insects. The field cage contained a tomato plant, which had just started flowering, in a 5 gallon pot and two sources of 10% sucrose solution for the moths to feed upon.

Individual female moths were placed in holding cages on the floor of the field cage. When a female began to initiate flight, she was released from the holding cage and the observations began. One investigator maintained constant visual contact with the moth while the second investigator recorded each time the moth made tarsal contact with a leaflet, the location of that leaflet, and if the female curled her abdomen and made contact with her ovipositor. These behaviors were recorded on a diagram of the tomato plant. The experiments ended after the female had remained sedentary for 20 min. A total of six trials were run.

Once a trial was completed, the plant was immediately taken to the laboratory. The location of the eggs was recorded on the diagram and the eggs were removed.

To determine if leaflet position on the plant influenced oviposition after tarsal contact, the plants were visually divided into thirds. The middle and lower thirds were combined to form one group. A Chi-square test of independence was then performed to determine if the moths were more likely to lay on leaflets they touched from the top third of the plant versus leaflets from the other two-thirds of the plant.

Leaflet surface extracts of aqueous compounds were prepared after measuring the area. The tomato leaflets were cut at the base of the petiolule, and the area measured using SigmaScan for the PC. The leaflets were then dipped into 50ml of boiling diH_2O for 1 second, being careful not to dip the cut end (Zobel and Brown 1988). The leaf tissue was immediately placed in a zip lock bag and frozen with dry ice. Tissue samples were stored at -80°C and surface extracts were stored at -4°C.

In order to prepare the extracts for HPLC analysis, 2ml aliquots of the surface extract were dried in a rotovap. and resuspended in 0.2mls of ddiH_2O. The solution was run through a PRP-1 column and the column was washed with an additional 0.2ml of ddiH_2O.

The amounts of fructose and inositol present in the surface wash were measured using a Dionex (Sunnyvale, CA) HPLC system in conjuction with a HPX-87C Aminex column with H_2O at 85°C equipped with a Gison (Middleton, WI) model 302 pump. Carbohydrate detection was by pulsed amperometric detection (Dionex Advanced PAD) at 35°C. Detection limits for inositol were at least 10 pmoles/50ul sample (Adams *et al.* 1993).

A Bradford assay as modified by Jones *et al.* (1989) was used to measure the protein levels of the leaflets. The leaflets were ground in liquid nitrogen and 100mg of the tissue was then ground in 1ml of 0.1N NaOH. The grinding tube and pestle were washed with an additional 1ml of NaOH. The samples were vortexed for a few seconds and then left to extract at room temperature for 30-45 min. The supernatant was decanted and 100ul aliquots of three replicates per sample were mixed with 5mls of Bradford reagent (Bio-Rad). The absorbance at 595nm was recorded for each aliquot and calibrated against a standard curve made from egg albumin (Sigma).

Results

Egg-distribution

The number of eggs found on the tobacco, tomato, and basil plants on July 16 and July 17, 1993 are shown in Table 5.1. Although a higher percentage of eggs was laid on tobacco, it is not necessarily preferred since the available leaf area on tobacco was greater and replication does not allow statistical treatment. Tobacco and tomato were acceptable, whereas the non-host, basil, was not an acceptable host.

The intraspecific egg-distribution patterns for tobacco and tomato plants were also examined for the first experiment. The data was pooled for all plants within a species for each trial. The expected Poisson distributions versus the actual distributions were compared using a Chi-square goodness-of-fit test. The results for July 16 are shown in Figure 5.1 for tobacco and Figure 5.2 for tomato. The results for July 17 are shown in Figure 5.3, tobacco, and Figure 5.4, tomato. The data was pooled for all plants within a species for each night. In all cases the observed distribution patterns were significantly different from the expected patterns, indicating selectivity among leaves.

The results from the second experiment, in which a single female moth was presented with a single tomato plant, are shown in Figures 5.5 and 5.6. In neither trial did the observed egg-distribution pattern differ significantly from the expected, but in the second trial there was a trend towards the moth selecting some leaflets more than others. In the trial presented in Figure 5.5, the moth only laid 11 eggs. The moth laid 56 eggs in the trial presented in Figure 5.6. Several of the eggs laid in this trial were laid on the stem of the plant.

Significantly more eggs were laid on the top halves of the plants than the bottom halves. In the large cage experiments, 60% of the eggs found on tomato were oviposited on leaves on the top half of the plant. On tobacco, 70% of the eggs were laid on leaves on the upper part of the plant. Chi-square goodness-of-fit tests showed that significantly more eggs were laid on leaves on the upper part of the plants than expected (Table 5.2). Tobacco plant #1 was not used in this analysis due to it having a broken shoot.

Oviposition in relation to leaflet position, protein level, fructose level, and surface inositol level

The results of the observations on intraplant selection behavior of six ovipositing *M.sexta* showed four categories of selection behavior; flying towards host-plant, hovering, tarsal contact, and finally oviposition. First was the approach towards a host-

plant, which was apparently directed. The second behavioral category was noted once the moth was near a plant. It consisted of hovering within a few centimeters of the surface of the plant and at times short flights between different regions of the plant. At this point the moth could touch the leaf surface with her tarsi, the third type of assessment behavior. Finally, the moth curled her abdomen and oviposited. The female was always observed to lay an egg once its ovipositor touched the leaf surface. Thus, the moths do not appear to use their ovipositor for host-plant selection. In almost all cases a single egg was laid per contact of the ovipositor to the leaf surface.

The moths were observed making tarsal contact with the plants a total of 128 times. The moths touched leaves from the top third of the plants 68% of the time, middle leaves were contacted 23% of the time, and only 9% of the contacts were made on leaves from the lower thirds of the plants. Moths were more likely to land on the top third of the plant (87 contacts) than the bottom two-thirds (41 contacts). However, lower leaves had a much higher acceptance rate than the upper leaves (Table 5.3). Fifty-six percent of the contacts with leaves from the top third of the plant resulted in oviposition, whereas 92% of the contacts with leaves from the lower two-thirds of the plant resulted in oviposition.

Figure 5.7 shows the number of times a moth accepted or rejected a leaf in relation to the protein level of the leaf. It is clear from visual inspection of the figure that there is no consistent relationship. It is interesting that plants, and leaves within plants, differed considerably in protein levels, further suggesting that protein level is not important in selection.

Figure 5.8 shows the results of each individual trial for inositol. In no case was there an obvious relationship between oviposition and inositol levels. The six plants fell into two major categories; those with inositol levels always below 1 ug/cm^2, and those with more variable levels. In one case levels were up to 2.5 ug/cm^2, one up to 4.5 ug/cm^2 and the third up to 5.3 ug/cm^2.

Figure 5.9 shows the results of each individual trial for fructose. The results indicate no difference in the surface fructose levels of the leaflets that were accepted versus those that were rejected. Once again the plants, and the leaves within a plant, differed greatly in surface fructose levels.

Discussion

Results clearly show that the moths distribute their eggs in a non-random manner. The majority of the eggs were oviposited on leaves on the upper half of the tobacco and tomato plants. A much larger and more comprehensive study done by Madden (1945) in which he recorded oviposition sites from over one-thousand tobacco plants during a five year period, showed that *M. sexta* laid over 57% of their eggs on leaves from the top third of the plant and only 4% of their eggs on the leaves from the bottom third of the plant.

Nonrandom use of tissue within a plant can arise from the same categories of behavioral responses that influence nonrandom use of different conspecific hosts. The behavioral categories put forth by Papaj and Rausher (1987), and as modified for nonrandom intraplant use, include 1) nonrandom movement patterns within a host-plant; 2) nonrandom rates of contact with different plant parts; and 3) nonrandom selection subsequent to contact.

Although movement patterns within a host-plant were not recorded in this study, it did appear that the moths spent more time hovering near the top leaves of the plant without contacting the plant. On several occasions the moth hovered near a leaf, flew to another leaf and then returned to again hover near the first leaf. Non-random intraplant movements, if they truly are occurring would appear to be based on the visual and/or olfactory cues that the moth is receiving from the plant.

Nonrandom rates of contact with the leaves in different parts of the plant did occur. It appears that the preference for ovipositing on leaflets from the top third of the plant is based on the moth preferentially making tarsal contact with the top third of the plant. In the

observational studies, moths made tarsal contacts with the leaflets from the upper part of the plant 68% of the time.

The preference for contacting upper leaves may be due to the moths hovering more towards the top of the plants and thus having more opportunity to contact the upper leaves. The upper, younger leaves, may also be emitting more volatile than the lower leaves and are thus more attractive than the lower leaves. This has been shown in other plant species. For example young *Eucalyptus* leaves were found to have higher rates of monoterpene and isoprene emissions than the older leaves (Guenther, *et al.* 1991). Another possibility is that it is physically easier for the moth to make tarsal contact with the leaves near the top of the plant.

By ovipositing mainly on leaves from the top third of the plant, the moths are selecting tissue that have on average the highest levels of protein (Chapter 6). However, the leaf analysis showed that the individual moths are not selecting leaflets with high protein levels. There was no difference in the amount of protein within leaflets that the moth touched and accepted versus those touched and rejected. These results are not surprising since there is no known mechanism for the moths to taste protein directly (Bernays and Chapman 1994; p. 129). In addition, it appears that they are not using cues related to protein levels gained from tarsal contact.

Although the moths preferentially landed on leaves on the upper third of the plant, lower leaves had a much higher acceptance rate post tarsal contact. The higher acceptance rate for the lower leaves may be due to lower levels of secondary metabolites on the leaf surface. High levels of alkaloids have been shown to be highly correlated with high photosynthetic rates in some species of solanaceous plants (Baldwin 1994). So it could be adaptive for females to choose leaf tissue with lower alkaloid levels to protect the newly hatched larvae from deterrent or toxic compounds. Furthermore, the trichome density is

lower on the older, larger leaves. Thus, the larvae would be less likely to get stuck in the trichome exudates on the lower leaves.

The fructose levels on the tomato leaflet surfaces were not correlated with oviposition behavior of *M.sexta*. However, in another species of Lepidoptera, the European Corn Borer, *Ostrinia nubilalis*, fructose levels on the leaf surfaces of corn and sunflower were highly correlated with oviposition preference (Derridj *et al.* 1989).

As seen in Chapter 3, inositol is a phagostimulant for the third and fifth instar larvae, and it appears to be both a utilizable carbohydrate source and a necessary nutrient for the larvae. Thus, it was expected that the moths would prefer to oviposit on leaves with relatively high levels of inositol, especially since it has been found in other species of moths that larval and adult chemoreceptors have similar sensitivities to relevant chemicals (Blaney and Simmonds 1988). However, none of the present data indicate that inositol was an oviposition stimulant.

There are several possible reasons why these data show no effect of surface inositol on moth oviposition. One is simply that there really are no effects on *M.sexta* females; a conclusive answer probably requires more work on standardized plants with independent measures of plant physiological status. The variation among plants in this study is a cause for concern in this respect.

Diverse factors probably influence inositol levels in plants. Inositol levels are related to growth rates of plant tissue. Since inositol is so important in cell wall synthesis, membrane synthesis and second messenger systems levels might be expected to be higher in actively growing plants (see Chapter 1).

It is also known that *myo*-inositol acts as substrate for polysaccharide biosynthesis in response to some types of wounding (Loewus *et al.* 1990). The inositol that is not converted to the polysaccharides would most likely be leached to the surface of the leaf or leaflet. Thus, a high level of surface inositol may indicate a leaflet that has been previously

damaged. Damaged tissue is known to have relatively high levels of secondary chemicals that act as feeding deterrents for the young larvae and toxins (Baldwin 1988).

Inositol levels are also elevated in salt-stressed plants. Sacher and Staples (1985) found a steady increase in the level of *myo*-inositol when a tomato plant was salt stressed. In the leaves of the salt stressed plants, *myo*-inositol accounted for three-fourths of the soluble carbohydrates.

In conclusion, *M.sexta* moths oviposit upon leaves in a non-random manner. The majority of the eggs are laid on the leaves on the upper part of the plant most likely due to the moths spending more time hovering near the upper leaves than the lower leaves and to the moths making a greater number of contacts with leaves on the upper parts of the plants. However once tarsal contact has been established, the moths are more likely to lay eggs on leaves on the lower part of the plant. No evidence was obtained for the ability of the moth to discriminate in relation to protein, surface fructose or surface inositol.

Table 5.1 The number of eggs laid by *M.sexta* on the tobacco, tomato, and basil plants on the nights of July 16, 1993 and July 17, 1993. Five female moths were placed in the field cage on July 16 and 3 female moths were present on July 17. The moths accepted the two host-plants, tobacco and tomato, but rejected the non-host, basil.

	Tobacco			Tomato		Basil	
	Plant #1	Plant #2	Plant #3	Plant #1	Plant #2		Totals
July 16, 1993	22	92	72	24	57	0	267
July 17, 1993	177	81	126	101	76	3	564

Table 5.2 Number of eggs laid by *M.sexta* on the top and bottom halves of tomato and tobacco plants in a field cage. Chi-square goodness-of-fit tests showed that significantly more eggs were laid on leaves on the upper half of the plant than expected in 6 of the 8 cases. Tobacco plant # 1 was removed from the analysis due to it having a broken stalk.

			# of Eggs			
Species	Date	Plant #	Top half	Bottom half	X^2	P
Tomato	7/16/93	1	19	5	8.16	<0.05
Tomato	7/17/93	1	66	35	9.52	<0.005
Tomato	7/16/93	2	38	19	6.33	<0.05
Tomato	7/17/93	2	42	34	0.84	NS
Tobacco	7/16/93	2	37	27	1.56	NS
Tobacco	7/17/93	2	48	23	31	<0.001
Tobacco	7/16/93	3	46	26	5.55	<0.05
Tobacco	7/17/93	3	92	34	26.7	<0.001

Table 5.3 Moths were more likely to oviposit on the leaflets on the bottom half of the plant than those on the top half a tomato leafle once tarsal contact was established.

	Accept	Reject	Totals
Top half	49	38	87
Bottom half	38	3	41
Totals	87	41	128

Fig.5.1 The actual egg-distribution frequencies versus the expected, Poisson distribution, frequencies for the tobacco plants used the in July 16, 1993 large, screened-cage experiment. The expected frequencies were significantly different from the observed frequencies ($X2=151$, $p<0.0001$).

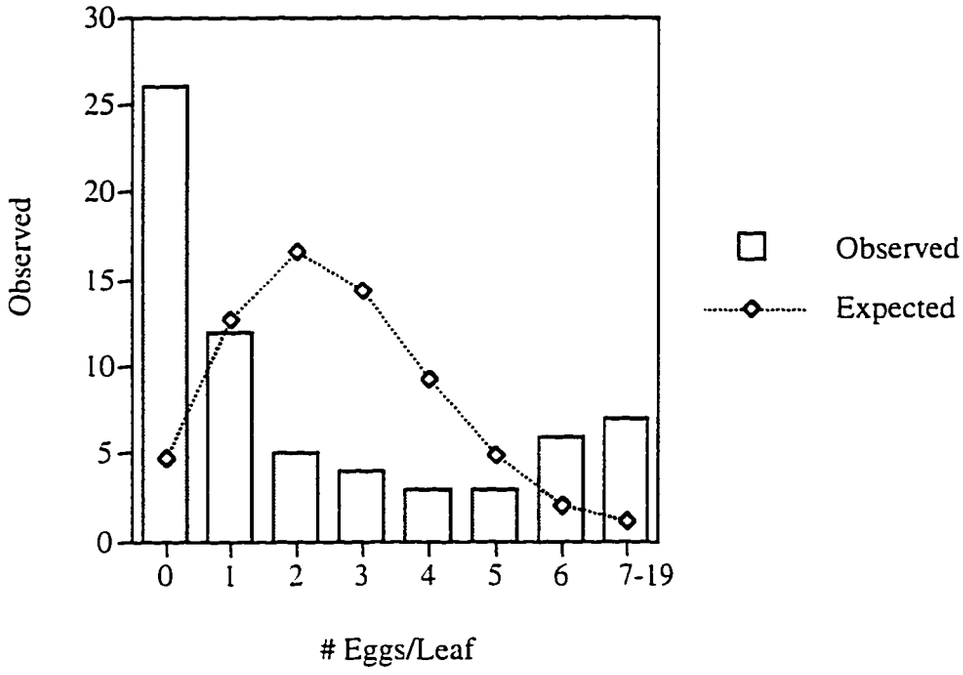


Fig. 5.2 The actual egg-distribution frequencies versus the expected, Poisson distribution, frequencies for the tomato plants used the in July 16, 1993 large, screened-cage experiment. The expected frequencies were significantly different from the observed frequencies ($X2=15.38$, $p<0.005$).

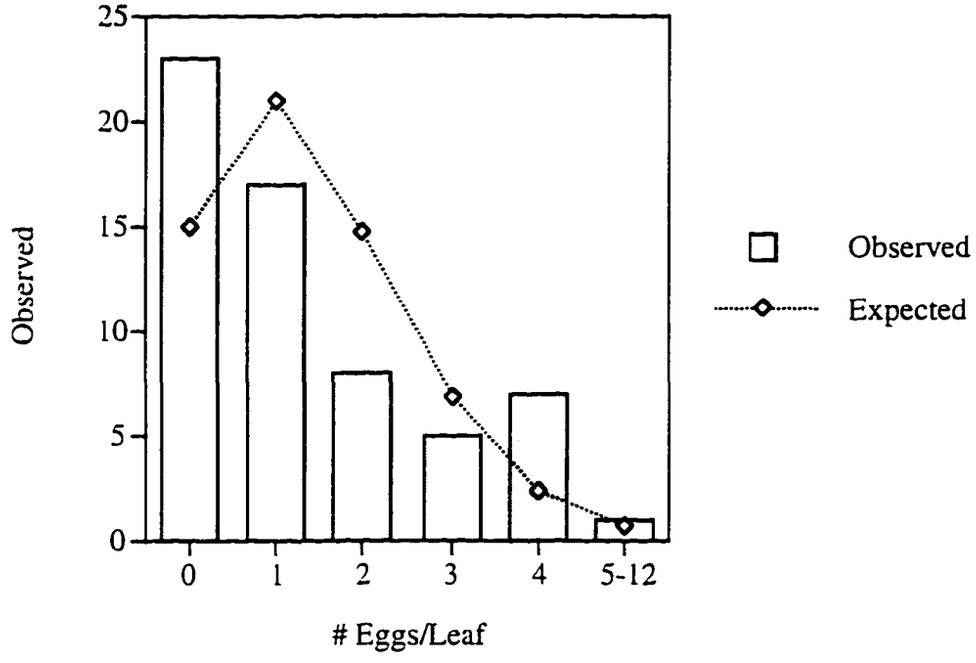


Fig. 5.3 The actual egg-distribution frequencies versus the expected, Poisson distribution, frequencies for the tobacco plants used the in July 17, 1993 large, screened-cage experiment. The expected frequencies were significantly different from the observed frequencies (Chi-squared=2.58, $p<0.0001$).

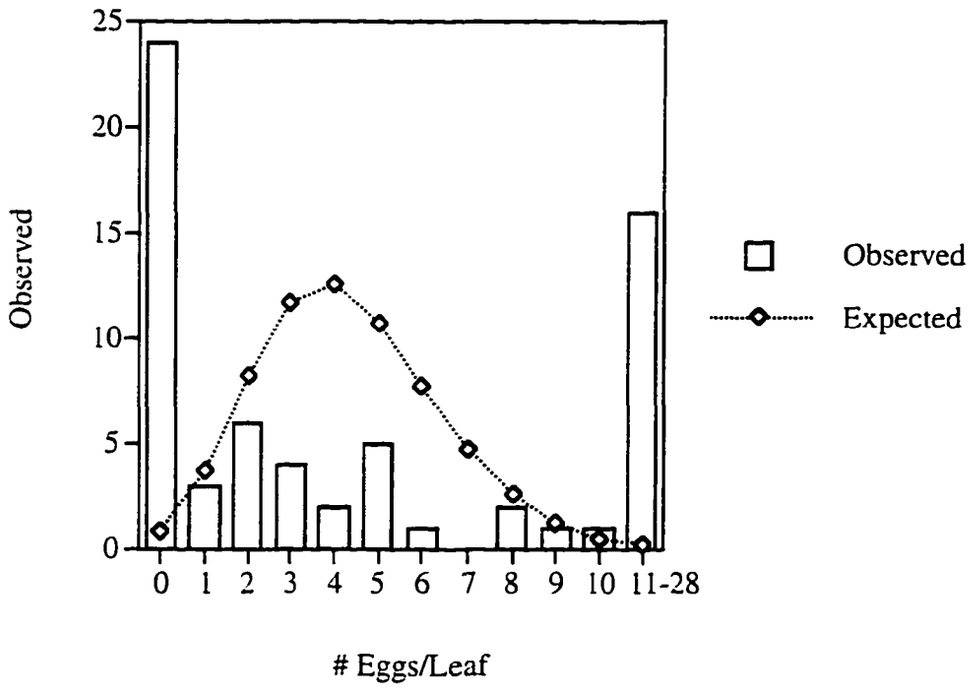


Fig. 5.4 The actual egg-distribution frequencies versus the expected, Poisson distribution, frequencies for the tomato plants used the in July 17, 1993 large, screened-cage experiment. The expected frequencies were significantly different from the observed frequencies (Chi-squared=61.8, $p<0.0001$).

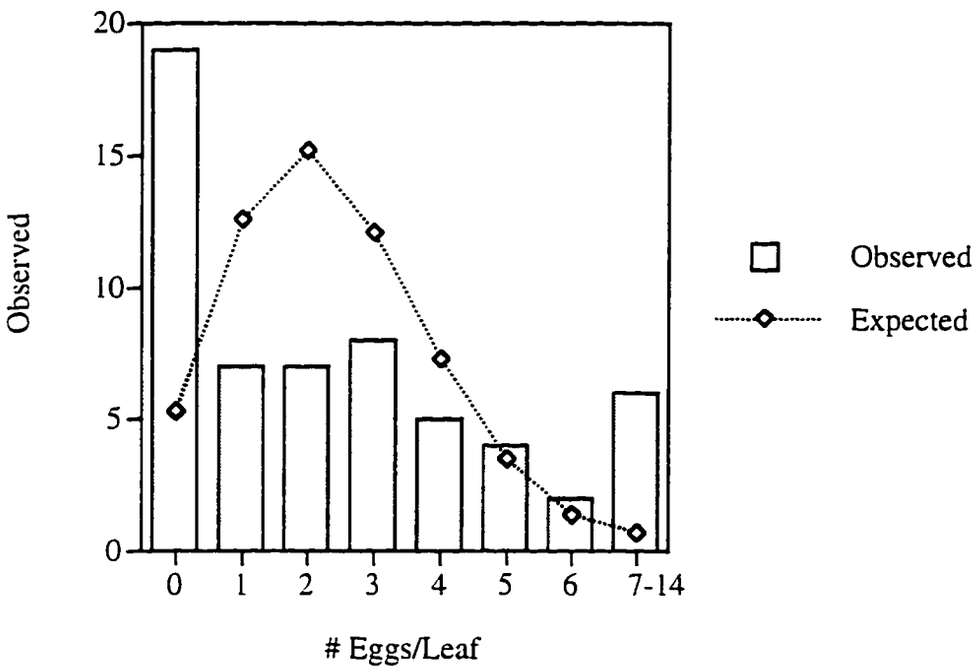


Fig. 5.5 The actual egg-distribution frequencies versus the expected, Poisson distribution, frequencies for the tomato plants used the in July 16, 1993 small-cage experiment #1. In this experiment a single female moth was presented with a single tomato plant. The observed frequencies were not significantly different from the expected frequencies (Chi-squared=3, p=0.22).

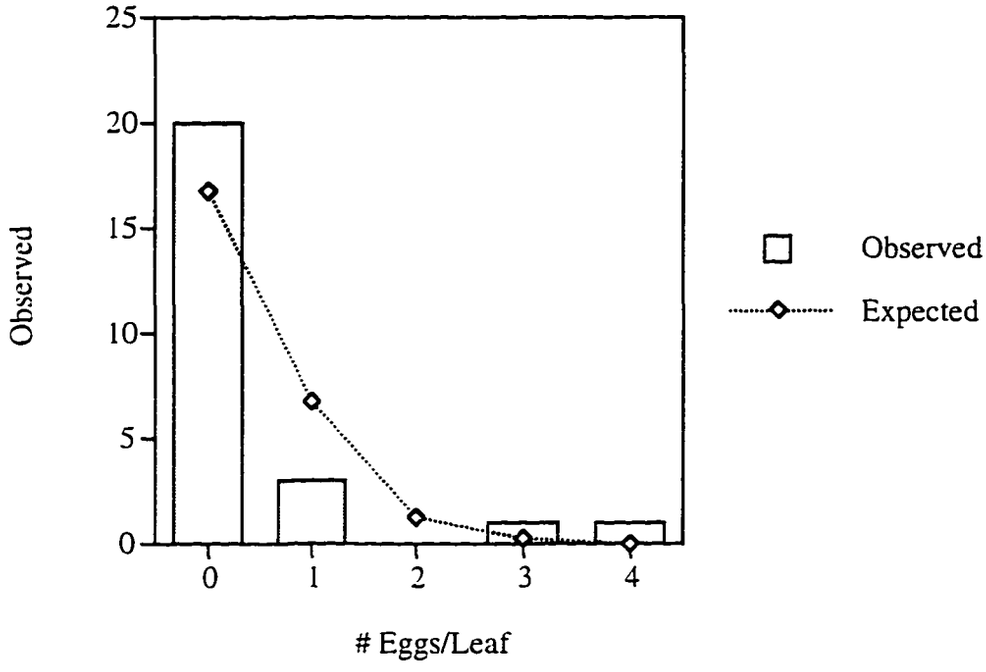


Fig. 5.6 The actual egg-distribution frequencies versus the expected, Poisson distribution, frequencies for the tomato plants used the in July 16, 1993 small-cage experiment #2. In this experiment a single female moth was presented with a single tomato plant. The observed frequencies were not significantly different from the expected frequencies, but there was a trend towards there being a difference (Chi-squared=7.8, $0.05 < p < 0.1$).

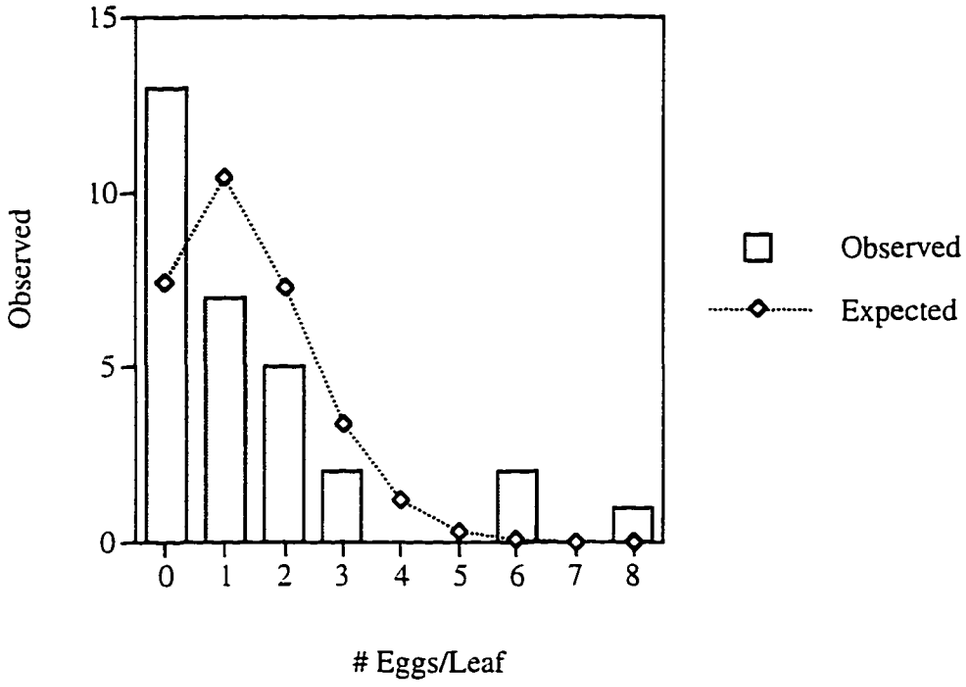


Figure 5.7
The relationship between protein levels of the leaflets and moth acceptance behavior. For each trial a different moth and plant was used. Each tarsal contact was recorded as either an acceptance (oviposition) or rejection (tarsal contact established but no oviposition). A leaflet may be counted more than once. Note that some crosses have a bold horizontal line indicating more than one point.

Fig. 5.7

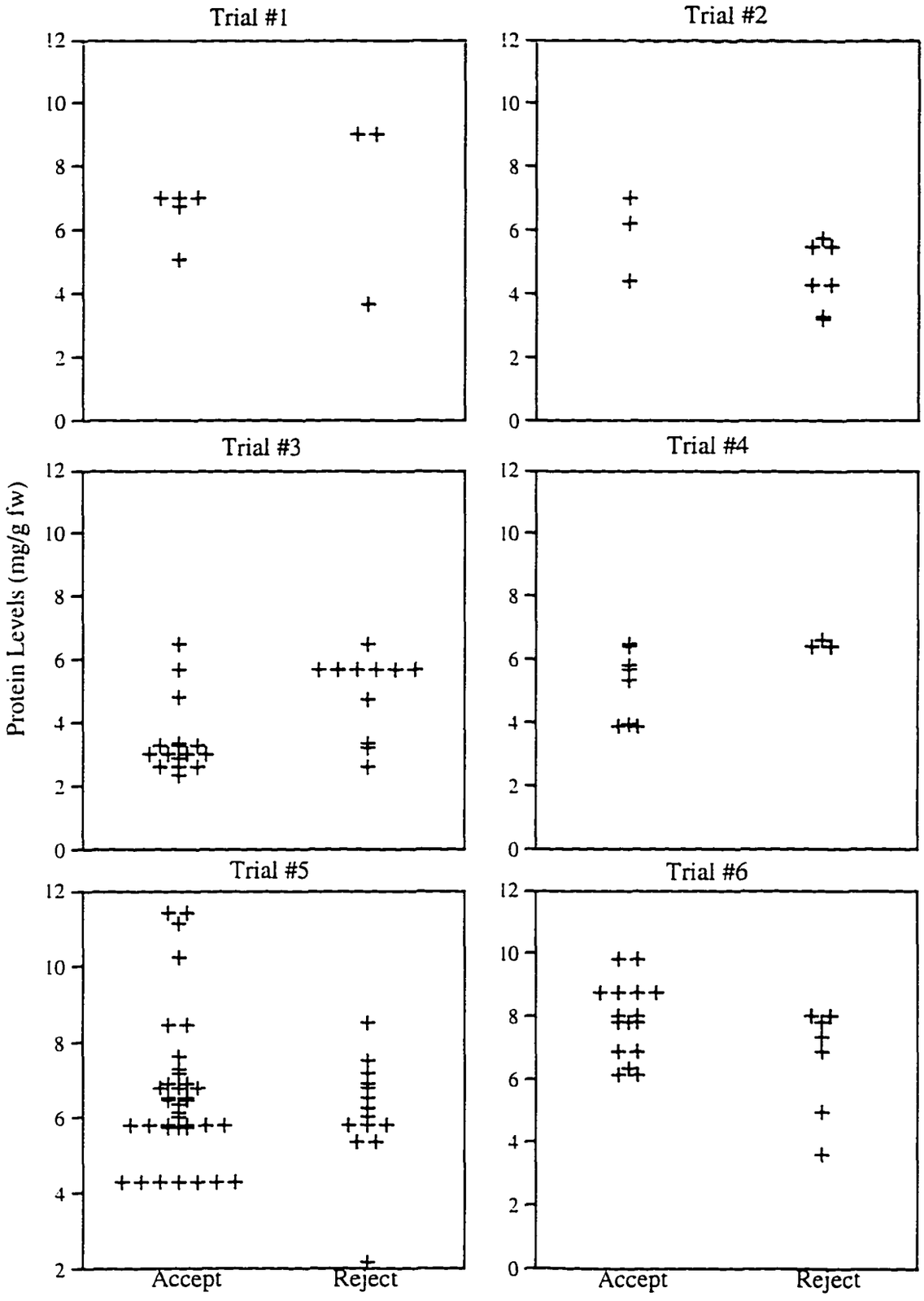

Trial #1
Trial #2
Trial #3
Trial #4
Trial #5
Trial #6
Protein Levels (mg/g fw)
0
2
4
6
8
10
12
Accept
Reject

Figure 5.8
The relationship between surface inositol levels of the leaflets and moth acceptance behavior. For each trial a different moth and plant was used. Each tarsal contact was recorded as either an acceptance (oviposition) or rejection (tarsal contact established but no oviposition). A leaflet may be counted more than once. Note that some crosses have a bold horizontal line indicating more than one point.

Fig. 5.8

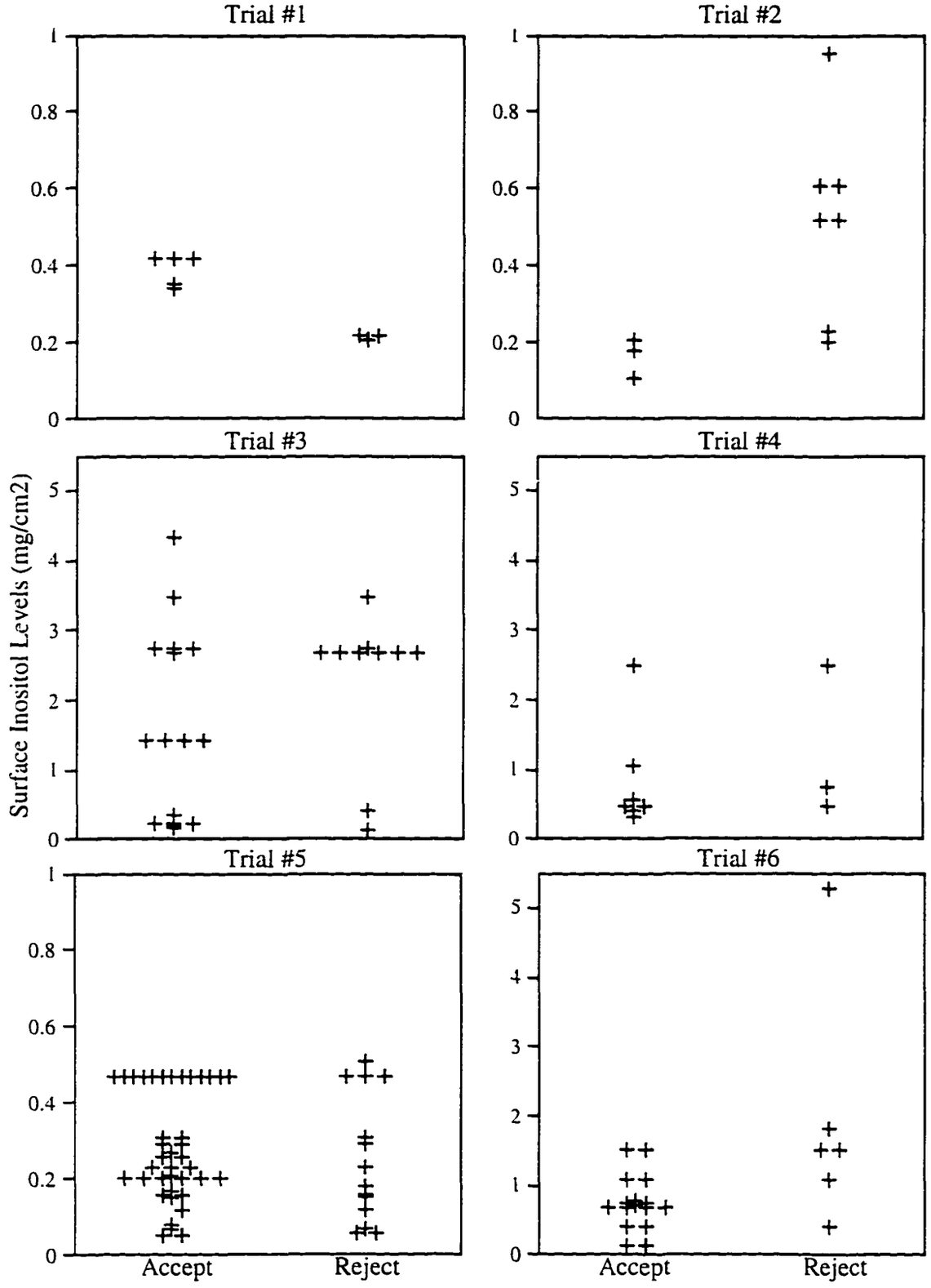
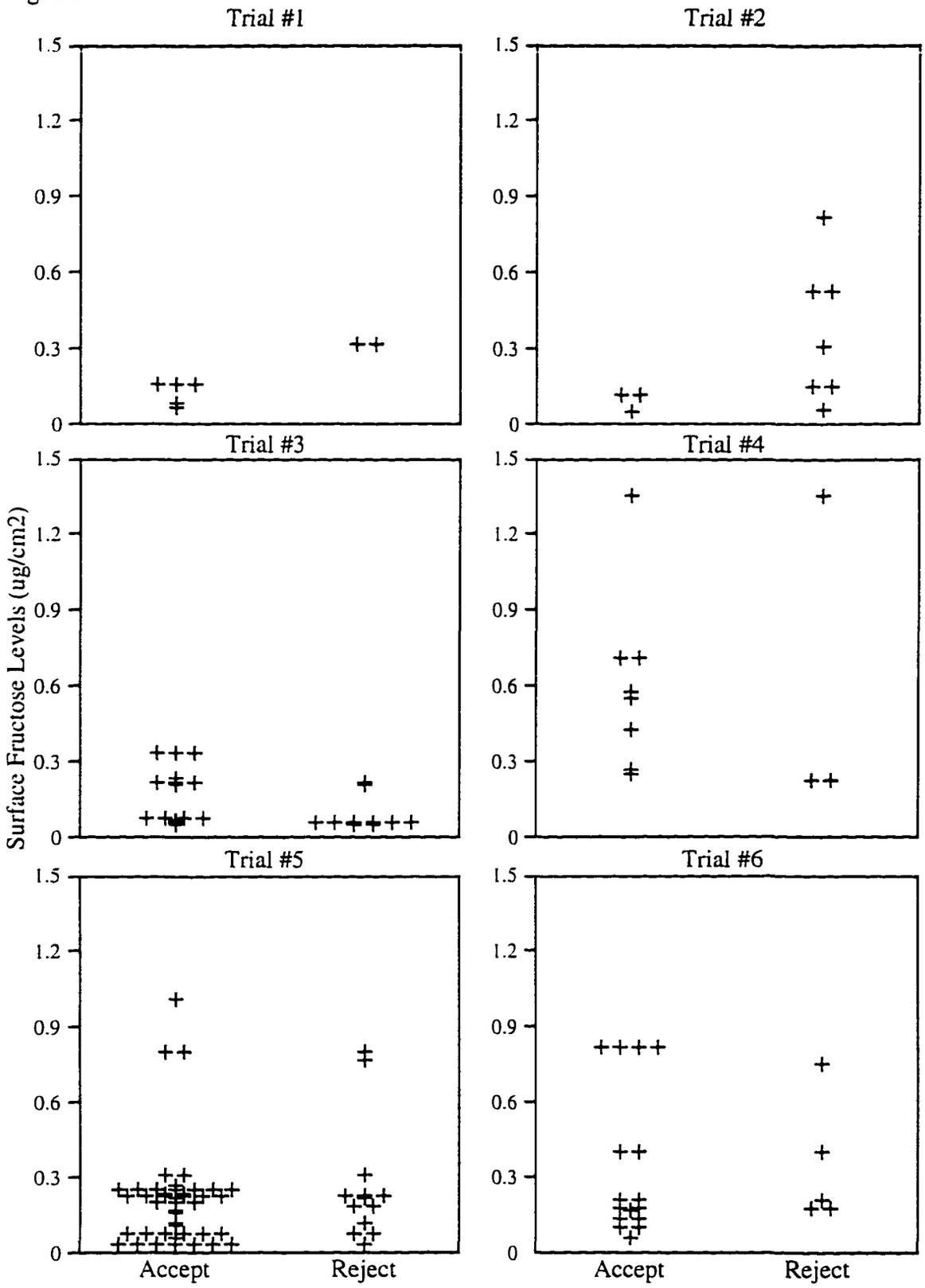

Trial #1
Trial #2
Trial #3
Trial #4
Trial #5
Trial #6
Surface Inositol Levels (mg/cm2)
Accept
Reject
Accept
Reject

Figure 5.9
The relationship between surface fructose levels of the leaflets and moth acceptance behavior. For each trial a different moth and plant was used. Each tarsal contact was recorded as either an acceptance (oviposition) or rejection (tarsal contact established but no oviposition). A leaflet may be counted more than once. Note that some crosses have a bold horizontal line indicating more than one point.

Fig. 5.9


Trial #1
Trial #2
Trial #3
Trial #4
Trial #5
Trial #6
Surface Fructose Levels (ug/cm2)
1.5
1.2
0.9
0.6
0.3
0
Accept
Reject

CHAPTER 6: INOSITOL, SUGAR, AND PROTEIN LEVELS IN LEAVES FROM TWO SPECIES OF SOLANACEOUS PLANTS, *NICOTIANA TOBACUM AND LYCOPERSICON ESCULENTUM*

Introduction

Leaf inositol levels may serve as a cue to tissue quality. As mentioned in Chapter 1, inositol is a growth factor for plants, and is a precursor for second messengers, cellular membrane components, and cell wall components. Thus high inositol levels might indicate young, actively growing plant tissue. Young actively growing plant tissue has relatively high protein levels, due to the high levels of enzymes associated with photosynthesis especially RUBISCO. Since insects have no known mechanism for directly sampling the levels of protein in leaf tissue, it was hypothesized that leaf inositol levels may serve as a cue for caterpillars to select leaves for their tissue quality.

The first section of the final research chapter will focus on the levels of nutrients in the leaf tissue from two species of Solanaceous plants, tobacco, *Nicotiana tobacum*, and tomato, *Lycopersicon esculentum*.. In these experiments leaf tissue was sampled for inositol and protein levels to determine if the two were correlated. Inositol and protein levels were also examined in relation to leaf age and to individual plants.

The second set of experiments examines the levels of carbohydrates on the surface of the leaves along with the levels within the leaf tissue to determine if the two are correlated. Carbohydrate, especially sugar, is also a major nutrient needed by larvae. However, egg-laying moths can only taste the surface of the leaves. Thus, the goal of these experiment was to determine if insects could use the levels of the sugars on the surface as cues for carbohydrate levels available in the tissue.

Methods

The experiments were run in two series as follows:

Series 1: total protein vs inositol in tomato

total protein vs sugars in tomato

leaf age vs inositol and protein in tomato

total protein vs inositol in tobacco

leaf age vs inositol and protein in tobacco

Series 2: Multiple comparisons were made among the following in both tomato and tobacco:

surface levels of the sugars; sucrose, fructose and glucose

surface levels of inositol

tissue levels of protein

tissue levels of the sugars; sucrose, fructose and glucose

tissue levels of inositol

leaf age

Series 1

Comparison of inositol, sugar and protein levels in tomato leaf tissue

To determine if inositol levels could be a possible cue for protein levels in leaf tissue, the levels of inositol and protein in the leaves of tomato, *Lycopersicon esculentum*-Better Boy, were measured. The levels of the sugars; sucrose, fructose, and glucose were also measured. The five tomato plants used in this study were purchased from a local nursery.

All of the plants had five fully expanded leaves. One of the two most distal opposite leaflets from each leaf was measured for protein level and the other for inositol

and sugar levels. A third leaflet from each leaf was used for fresh and dry weight measurement to estimate water content.

The leaflets were harvested on July 14, 1993. For chemical analyses leaflets were individually placed in resealable plastic bags and immediately frozen on dry ice. The leaflets were stored at -80°C for up to 3 weeks. For water content, leaflets were quickly weighed, oven dried in open paper bags overnight at 50°C, and then reweighed.

The amounts of protein, inositol, glucose, fructose, and sucrose, were measured as described below. Using the regression equation generated from the water content analysis (Fig. 6.1), the leaf weights were converted to dry weights, and data were analyzed on a dry weight basis. One-way Analyses of Variance (ANOVA) were used to test for differences in sugar, inositol and protein levels among plants. A correlation matrix analysis along with a Fisher's r to z test was run to determine if the correlations among the sugars, inositol, and protein levels were significantly different from zero. Finally, leaves were numbered from the bottom of the plant to the top and a correlation matrix along with a Fisher's r to z test was run to determine if leaf number, a rough estimate of leaf age, was correlated with protein or inositol levels. Since the lower leaves also had a lower number, a positive correlation would mean that inositol or protein levels were higher in the younger leaves.

Comparison of inositol and protein levels in tobacco leaves

Leaves from three tobacco plants were harvested on August 8, 1993 for protein and inositol analysis. The tobacco plants had been grown from seed in the University of Arizona, Department of Entomology greenhouse. Each tobacco plant had between fifteen and twenty leaves.

The plants were divided from the top down into six parts. One leaf from each part was taken for analysis. The leaves were cut at the petiole and placed in individual, resealable plastic bags. The leaves were immediately frozen on dry ice and stored at -80°C for less than two weeks.

The leaves were analyzed on a fresh weight basis for protein and inositol content as described below. A one-way ANOVA was used to determine if there were differences among plants. A correlation analysis was also run to determine if leaf position on a plant correlated with protein and/or inositol levels. A correlation matrix analysis along with a Fisher's r to z test was run to determine if the correlation between inositol level and protein levels differed from zero. Also a correlation matrix was used to determine if inositol or protein levels were correlated with leaf number.

Series 2

Comparison of protein and tissue and surface levels of inositol and sugars in tomato and tobacco plants

The purpose of this analysis was to determine:

1) if leaf tissue and surface levels of sugars are correlated;

2) if either surface or tissue levels of inositol are correlated with leaf protein levels; and

3) if leaf number (which is a rough estimate of leaf age) is correlated with protein or sugar levels found in the leaf or the sugar levels on the surface of the leaf.

For this study, all plants were grown from seed in the Biochemistry departmental greenhouses at the University of Arizona, Tucson. They were in 1-gallon pots. Each tomato plant had between 11 and 14 fully expanded leaves, and were starting to flower.

On March 14, 15 and 28, 1994, leaflets from three, 10-to-12-week-old tomato plants were harvested. Three leaflets were taken from each leaf for individual analysis. All of the tobacco plants had between 10 and 15 fully expanded leaves and three had initiated flowering. Leaves from four, 8 to 10 week old tobacco plants were harvested on December 16 and 20, 1993.

To measure the amount of inositol and sugar on the leaf surface, a surface extract was made (see below). The tissue protein, inositol and sugar levels were also determined (see below). Individual sugars and inositol levels, and each as a percent of total sugar including inositol were compared. A correlation matrix along with a Fisher's r to z test was used to determine if the tissue levels and surface levels of each sugar and of inositol were correlated to one another. This same analysis was also used to determine if surface or tissue levels of inositol or sugars were correlated with tissue protein levels. A correlation analysis was also used to determine if leaf number correlated with the sugar, inositol or protein levels.

Surface extraction procedure

A surface extraction procedure was carried out to examine the levels of inositol and other sugars on the leaf surface. Zobel and Brown (1988) tested various methods for producing surface extracts from cabbage plants that would elicit a positive oviposition response by *Pieris rapae.* A one-second dip in boiling water procedure elicited the best response and at the same time prevented tissue components from leaching out of the cells. This method was presumed to be the best technique for surface extraction of water-soluble compounds.

Tobacco leaves were cut at the base of the petiole, and their areas measured using SigmaScan for the PC. The leaves were then dipped into 200 mls boiling diH_2O for 1 second, being careful not to dip the cut end. Tomato leaflets were cut at the base of the petiolule. Each tomato leaflets was separately dipped into 50 mls of boiling diH_2O for one second (Zobel and Brown 1988). The leaflet was then immediately placed in a zip lock bag and frozen with dry ice. The surface-extracted leaflets or leaves to be used for tissue analysis were stored at -80°C and surface extracts were stored at -4°C for up to three months.

In order to prepare the extracts for HPLC analysis, 2 ml aliquots of the surface extract were dried in a rotovap. and resuspended in 0.2 mls of ddiH_2O. The solution was run through a PRP-1 column and the column was washed with an additional 0.2 mls of ddiH_2O.

Tissue extraction procedure for inositol and sugars

The procedure used to extract sugars and inositol from the leaf tissue was developed by Adams *et al.* (1993). Plant tissue was ground with a mortar and pestle in liquid nitrogen. A 50 mg aliquot was then ground in a 1.0 ml solution of 12 parts ethanol, 5 parts chloroform and 3 parts water. Another ml of ddiH_2O was added to the solution and the solution was vortexed. After precipitation of solids, 0.2 mls of the supernatant was dried in a rotovap. This was resuspended in 0.2 mls of ddiH_2O and filtered through a PRP-1 column. The column was washed with an additional 0.2 mls of ddiH_2O.

HPLC analysis

The analysis of soluble sugars and sugar alcohols was performed using a Dionex (Sunnyvale, CA) HPLC system in conjunction with a HPX-87C Aminex column with H_2O at 85°C equipped with a Gison (Middleton, WI) model 302 pump. Carbohydrate detection was by pulsed amperometric detection (Dionex Advanced PAD) at 35°C. Detection limits for each sugar were at least 10 pmoles/50 ul sample. For calibration standards, various sugars from different sources were used (Adams *et al.* 1993).

Protein analysis

A Bradford assay as modified by Jones *et al.* (1989) was used to measure the protein levels of the leaves and leaflets. The leaves or leaflets were ground in liquid nitrogen and 100 mg of the tissue was then ground in 1 ml of 0.1 N NaOH. The grinding tube and pestle were washed with an additional ml of NaOH. The samples were vortexed for a few seconds and then left to extract at room temperature for 30-45 min. The supernatant was decanted. Aliquots of 100 µl were mixed with 5 ml of Bradford reagent

(Bio-Rad). For each sample three replicates were run. The absorbance at 595 nm was recorded using a Milton Roy Spectronic 1201 UV spectrophotometer for each aliquot and calibrated against a standard curve made using egg albumin (Sigma).

Results

Series 1:

Comparison of inositol, sugar and protein levels in tomato leaf tissue

Actual concentrations of protein, inositol, sucrose, fructose, and glucose for the five plants are shown in Fig. 6.2 a-e. The mean amount of protein found in the tomato leaves was 113±9 mg/g dw. However, protein levels varied greatly both within plant and among plants. There was almost a 10-fold difference between the lowest and highest level of protein.

Inositol levels on average were 1.2±0.17 mg/g dw. Again there was a great deal of variation. Two of the plants had less than 1 mg/g dw in all of the foliage tested whereas the other plants had foliage with more than 1.5 mg/g dw in most of the leaflets tested.

On average, the most abundant sugar was fructose (5.0±0.85 mg/g dw) followed by glucose (3.4±0.38 mg/g dw) and then sucrose (3.1±0.39 mg/g dw). In each of the sugars tested there was again almost a 10-fold variation between the lowest and highest levels.

Results of the ANOVA (Table 6.1) showed a plant effect for all of the sugar levels and the inositol levels. The ANOVA also suggested a trend for a plant effect on the protein levels ($p=0.06$).

Results of the correlation matrix and the Fisher's r to z test for the tomato leaves are show in Table 6.2. The results show inositol, sucrose and protein levels are correlated with one another in tomato leaves. Finally, leaf number is correlated to both inositol

(correlation coefficient = 0.408, $p<0.05$) and protein levels (correlation coefficient = 0.668, $p<0.0005$). The top leaves had the highest levels of protein and inositol.

Comparison of inositol and protein levels in tobacco leaves

Actual concentrations of protein and inositol found in the three plants are shown in Figures 6.3 a & b. In these plants the mean level of protein was 11.3±1.6 mg/g fw. Again there was 10-fold variation between the lowest and highest concentrations of protein. The mean level of inositol was 0.92±0.12 mg/g fw.

The results of the ANOVA (Table 6.3) did not show a plant effect on either protein or inositol levels. Protein levels were also correlated with leaf position on the plant. The leaves near the top of the plants had higher levels of protein than those near the bottom. Protein and inositol levels were positively correlated with one another in the tobacco plants tested (Table 6.4).

Series 2:

Comparison of tissue and surface levels of inositol and sugars in tomato plants

Amounts of the compounds present are shown in Fig. 6.4 a-e. Tissue protein levels averaged 5.75±0.22 mg/g fw (mean±S.E.), but there was a great deal of within plant variation. The most abundant sugar in the tissue of the leaves was fructose (1.14±.11 mg/g fw) followed by glucose (0.55±0.08 mg/g fw) and then sucrose (0.43±0.04 mg/g fw). Tissue inositol levels were 0.76±0.05 mg/g fw.

On the surface of the leaf inositol was more abundant than any of the sugars (0.30±0.03 mg/cm^2). Sucrose levels averaged 0.14±0.03 mg/cm^2. The level of fructose averaged 0.05±0.02 mg/cm^2 and the level of glucose averaged 0.04±0.01 mg/cm^2.

When tissue and surface levels of the sugars and inositol were compared, there was no correlation between the level of a sugar on the surface of a leaflet and the level found within the tissue (Table 6.5). However, the amount of inositol as a percent of all sugars including inositol was higher than expected. In the tissues it was 26% overall, while on the

surface it was 56%. Using percentages calculated in this way, relative levels of inositol in the tissues and on the surface were correlated (Correlation coefficient=0.449. P<0.0001).

The actual levels of inositol found in the leaflet tissue were not correlated with the leaflet protein levels in this study. Surface levels of inositol were also not correlated with protein levels (Table 6.6). The levels of inositol as a percentage of all the sugars, on both the surface of the leaflet and within the leaflet tissue, was correlated to protein levels (Table 6.6).

Leaf position was positively correlated with protein level (Correlation coefficient=0.352, p<0.01), thus the leaves near the top of the plant had more protein than those near the bottom. However, leaf position was negatively correlated with the level of inositol on the surface of the leaf (Correlation coefficient=-0.361, p<0.005). The position of the leaf was not correlated with any other factors measured.

Comparison of tissue and surface levels of inositol and sugars in tobacco plants

In tobacco, protein levels in this study were considerably lower than those in the previous study; 7.9±0.4 mg/g fw (Fig.6.5a). Inositol level in the tissue averaged 0.20±0.02 mg/g fw and on the surface, 0.15 mg/cm^2 (Fig.6.5b); lower than in the previous study. Sucrose was the most abundant sugar found in the leaf tissue (0.69±0.08 mg/g fw, mean±S.E.) (Fig.6.5c), followed by glucose (0.54±0.06 mg/g fw) (Fig.6.5d), and fructose (0.48±0.07mg/g fw) (Fig.6.5e). On the surface of the leaf sucrose was also the most abundant sugar (0.15±0.03 mg/cm^2) (Fig6.5c) followed by fructose (0.08±0.01 mg/cm^2) (Fig.6.5e) and then glucose (0.07±0.01 mg/cm^2) (Fig.6.5d).

For tobacco leaves, there was no correlation between the level of a sugar on the surface of the leaf and the level found within the leaf for any of the sugars tested or for inositol. When the data were transformed to percentages of the sugars present, there were correlations between the tissue and the surface of the leaf for the percentage of inositol and the percentage of fructose, but not glucose or sucrose (Table 6.7).

In this study protein levels were correlated only with leaf position. The leaves near the top of the plant, in other words the younger leaves, had a higher level of protein than those near the bottom (Correlation value 0.549, p=0.01). There was no correlation between inositol and protein levels (Table 6.8).

Discussion

General comparison between series

The two different series of experiments yielded different results in many respects. Also, comparison is complicated by the fact that levels of chemicals measured for tomato in series 1 was related to dry weight, whereas all others were on a fresh weight basis. Furthermore, plant developmental stage and growing conditions were different between the two series. Also, in series 2 the leaves were first dipped into hot water to remove the surface sugars, a procedure that may have affected the leaf water content and thus influenced results. There may have been extra water adhering to the surface, and different amounts of it on different aged leaves since trichome density decreases with leaf age.

With respect to tomato, rough comparisons between the two series can be made, assuming a water content of approximately 90% (Fig. 6.1). In series 2, protein concentrations were approximately 50% of those found in series 1, a figure that is reasonable, given that the plants were at a later stage of development and flowering had been initiated (Bernays and Chapman 1994). On the other hand, inositol levels were over five times higher in series 2 than in series 1. The three sugars measured were generally similar in series 1 and series 2.

With respect to tobacco, protein levels in series 2 were again about half those found in series 1. Inositol levels were also lower in series two (approximately one third of those found in series 1). Since sugars were not measured in series 1 a comparison of these cannot be made.

Leaf age effects

In all cases younger leaves had the higher levels of protein. Protein levels are known to vary among plants and also among tissues within the same plant. Within a plant the levels of protein are generally highest in the reproductive tissue and lowest in the older stems. Among leaves, it is usual for the protein levels to decline with age (Bernays and Chapman 1994). Most of the protein in the leaves is associated with photosynthesis. Ribulose bisphosphate carboxylase oxygenase (RUBISCO) an enzyme that fixes carbon during photosynthesis, can compose up to 50% of the soluble protein in young leaves. Younger leaves have more chloroplasts, higher levels of photosynthesis and thus more RUBISCO.

The levels of all three sugars and of inositol were variable, and no consistent patterns in relation to leaf age were observed.

Correlations among nutrients

In series 1, where the plants were younger and preflowering, positive correlations were found between inositol and protein levels in both tomato and tobacco leaves. In tomato, sucrose level was also correlated with protein level. Thus, for these plants it is conceivable that sucrose and particularly inositol, to which the larvae are so sensitive, could provide cues related to the availability of the important nutrient, protein. However, the correlations become weak in some cases when corrections are made for multiple comparisons, as in the case of inositol-protein comparison in tobacco.

In series 2, where plants were older and the sugars were first washed off the leaf surface, there was no correlation between inositol and protein levels. Neither were there correlations between levels of any of the sugars and the protein levels.

In order to get an overall view of whether inositol and protein levels are related, a Chi-squared test was run. The correlations between inositol and protein levels per plant were either positive or negative. The observed numbers of positive or negative correlations

were compared with an expected distribution of 50:50 if no overall correlation is expected. In 12 of the cases, there was a positive correlation and in three of the cases the correlation was negative. Comparing these numbers to the expected numbers, 7.5 in both cases, shows a significantly greater number of positive correlations than expected (Chi-square=5.4, $p<0.05$).

Comparison of tissue and surface levels of sugars and inositol

The lack of correlation between the levels of sugars found on the surface and within the tissue was surprising. This lack of correlation in some cases might be explained by the differences in the rates of leaching. Substances that are known to be leached from plants include organic materials such as sugars, sugar alcohols, and amino acids, as well as vitamins and secondary compounds (Tukey 1971). Inorganic nutrients are also leached from plant tissue in relatively large amounts. Leaf age and wettability are perhaps the two most important factors influencing the quality and quantity of the substances leached from the foliage. Young, actively growing tissue loses relatively little, whereas more mature tissue approaching senescence is very susceptible to leaching. The longer water stays in contact with the leaf surface, the more leaching occurs. Thus, the fact that young leaves are often more hydrophobic than older leaves maybe why younger leaves are more resistant to leaching.

Surface levels of inositol were extremely high in relation to the levels of other sugars. As discussed in the introduction of the thesis, inositol is an important precursor for pectic components external to the cell. If a large proportion of inositol is external to the cell it may be more readily leached to the surface than the sugars which are mainly intracellular or in the phloem. Another possible reason that inositol levels are high in relation to other sugars on the leaf surface is that the other sugars may be metabolized by organisms on the leaf surface more than inositol.

Conclusions

Protein levels were highest in young leaves as expected. However, inositol levels were much less predictable, and the relationship between inositol and protein is weak at best, although a significant relationship was found overall. More work is needed with better control over plant age and growing conditions to determine if these trends are widespread, and whether, in general it would be possible for insects to use inositol as a cue for the all-important levels of protein.

From this study, levels of sugars and inositol on the surfaces of the leaves could not predict tissue levels of these compounds, nor could they predict levels of protein in the tissue, so that even though they may be detected and stimulate feeding in larvae, they would not provide adult females with reliable cues of plant quality for their offspring.

Table 6.1 Differences occurred among the inositol, sucrose, glucose and fructose levels in tomato plants. Results of ANOVA are shown below. In all cases degrees of freedom were 4 for the plant and 20 for the residual.

	F-value	P-value
Protein	2.695	0.06
Sugars (total measured)	7.347	0.0008
Inositol	9.6	0.0002
Sucrose	7.62	0.0007
Glucose	3.8	0.0186
Fructose	3.6	0.0235

Table 6.2 Results of a correlation matrix for inositol, protein, and sugars in tomato leaf tissue. The results show a correlation between inositol and sucrose, inositol and protein, and protein and sucrose. Due to the number of comparisons, p has to be less than 0.0056 to be significant.

			Correlation	P-Value
		Inositol	0.745	<0.0001
		Sugars	0.321	0.12
Protein	X	Sucrose	0.763	<0.0001
		Fructose	0.008	0.97
		Glucose	0.279	0.18
		Protein	0.745	<0.0001
		Sugars	0.401	0.05
Inositol	X	Sucrose	0.813	<0.0001
		Fructose	0.097	0.65
		Glucose	0.299	0.15

Table 6.3 ANOVA results show no differences in protein or inositol levels in relation to the tobacco plants tested in series 1.

	F-value	P-value
Protein	0.097	0.91
Inositol	1.264	0.32

Table 6.4 Results of a correlation matrix for inositol, protein, and leaf number in tobacco plants from series 1. Leaves were numbered from the bottom to the top of the plant.

			Correlation	P-Value
Protein	X	Inositol	0.503	0.0462
Protein	X	Leaf Number	0.719	0.0011
Inositol	X	Leaf Number	0.359	0.1749

Table 6.5 Results of correlation studies comparing the levels of sugars and inositol found within the leaf tissue with the levels found on the surface of the leaves for tomato plants. The results showed a correlation between the leaf tissue and leaf surface for the percentage of inositol present.

			Correlation	P-Value
Tissue Inositol	X	Surface Inositol	-0.161	0.19
Tissue Sucrose	X	Surface Sucrose	0.022	0.86
Tissue Glucose	X	Surface Glucose	0.022	0.86
Tissue Fructose	X	Surface Fructose	0.052	0.67
% Inositol Tissue	X	% Inositol Surface	0.449	<0.0001
% Sucrose Tissue	X	% Sucrose Surface	-0.106	0.39
% Glucose Tissue	X	% Glucose Surface	0.228	0.06
% Fructose Tissue	X	% Fructose Surface	0.235	0.05

Table 6.6 Results of correlation studies between inositol and protein levels in tomato plants. The protein levels were correlated with the percentage of inositol present in the leaf tissue. The percentage of inositol present was calculated by adding the total amounts of all of the sugars that were measured and inositol present and then finding the percent of each sugar and inositol present.

			Correlation	P-Value
Tissue Inositol	X	Protein	-0.177	0.15
Surface Inositol	X	Protein	0.041	0.74
% Inositol Tissue	X	Protein	0.383	0.0006
% Inositol Surface	X	Protein	0.304	0.01

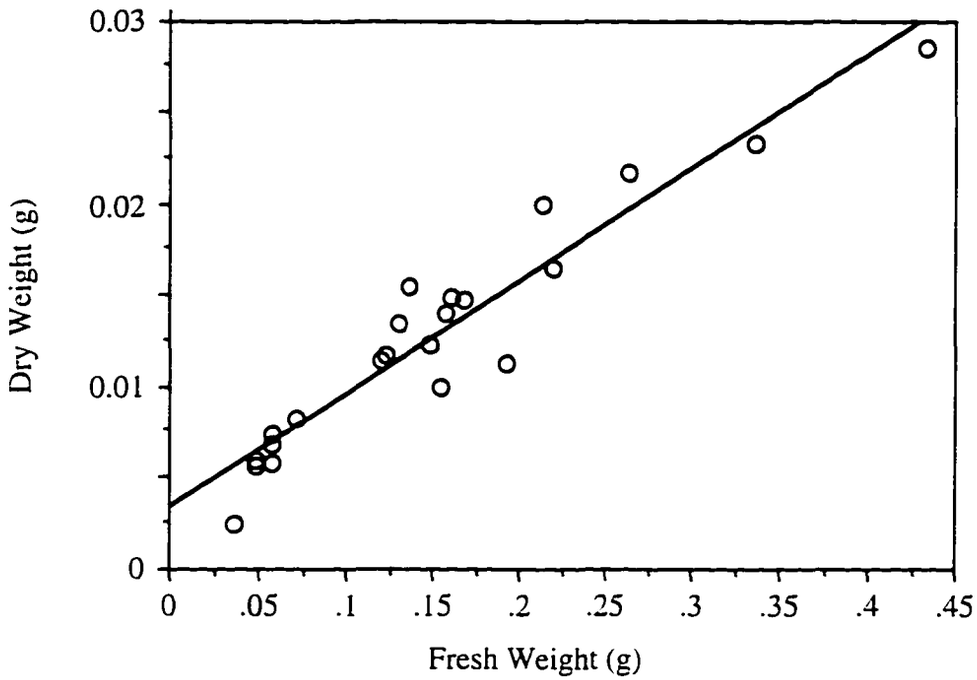
Table 6.7 Results of correlation studies comparing the levels of sugars and inositol found within the leaf tissue with the levels found on the surface of the leaves for tobacco plants. The results showed a correlation between the leaf tissue and leaf surface for both the percentage of inositol present and the percentage of fructose present.

			Correlation	P-Value
Tissue Inositol	X	Surface Inositol	-0.002	0.99
Tissue Sucrose	X	Surface Sucrose	0.023	0.91
Tissue Glucose	X	Surface Glucose	0.367	0.07
Tissue Fructose	X	Surface Fructose	0.356	0.08
% Inositol Tissue	X	% Inositol Surface	0.720	<0.0001
% Sucrose Tissue	X	% Sucrose Surface	-0.020	0.93
% Glucose Tissue	X	% Glucose Surface	0.273	0.06
% Fructose Tissue	X	% Fructose Surface	0.588	0.0016

Table 6.8 Results of correlation studies between inositol and protein levels in tobacco plants used in series 2. There were no correlations between inositol and protein levels.

			Correlation	P-Value
Tissue Inositol	X	Protein	0.174	0.41
Surface Inositol	X	Protein	-0.432	0.03
% Inositol Tissue	X	Protein	-0.221	0.29
% Inositol Surface	X	Protein	-.171	0.42

Fig. 6.1 Regression equation for converting the fresh weights of the tomato leaves used in series 1 to dry weights.



$Y = 3.428E\text{-}3 + .062 * X;\ R^2 = .908$

Fig. 6.2A Protein levels in tomato leaves from 5 young plants

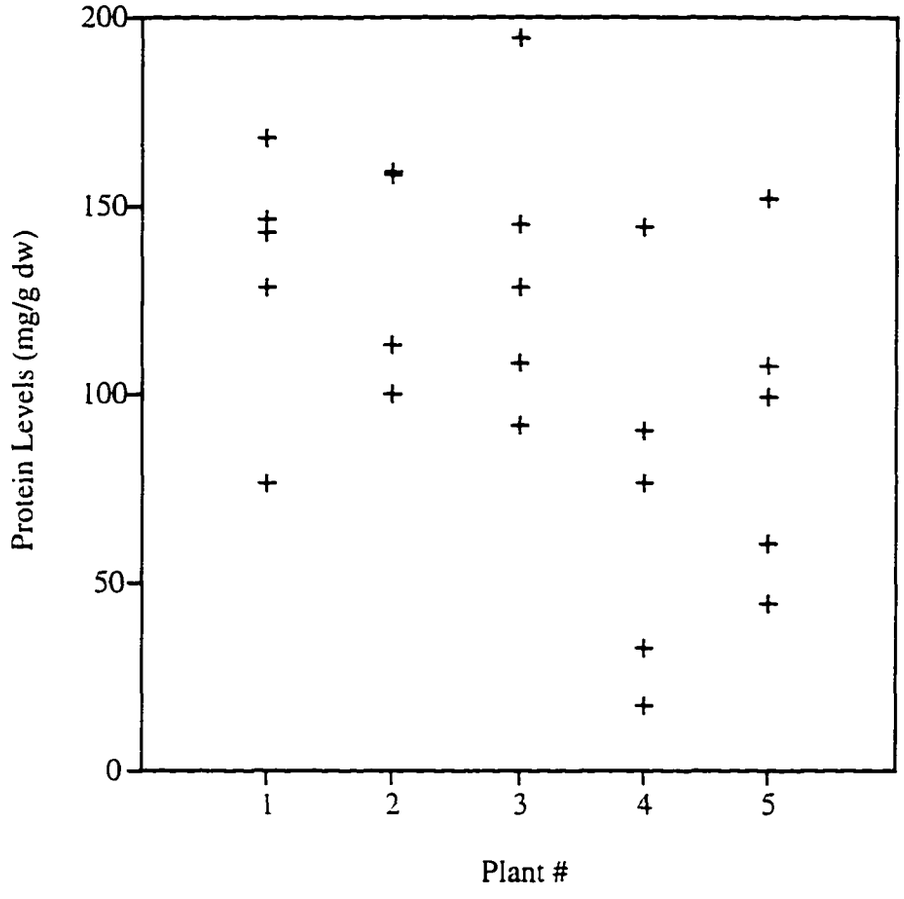


Fig. 6.2B Inositol levels in tomato leaves from 5 young plants

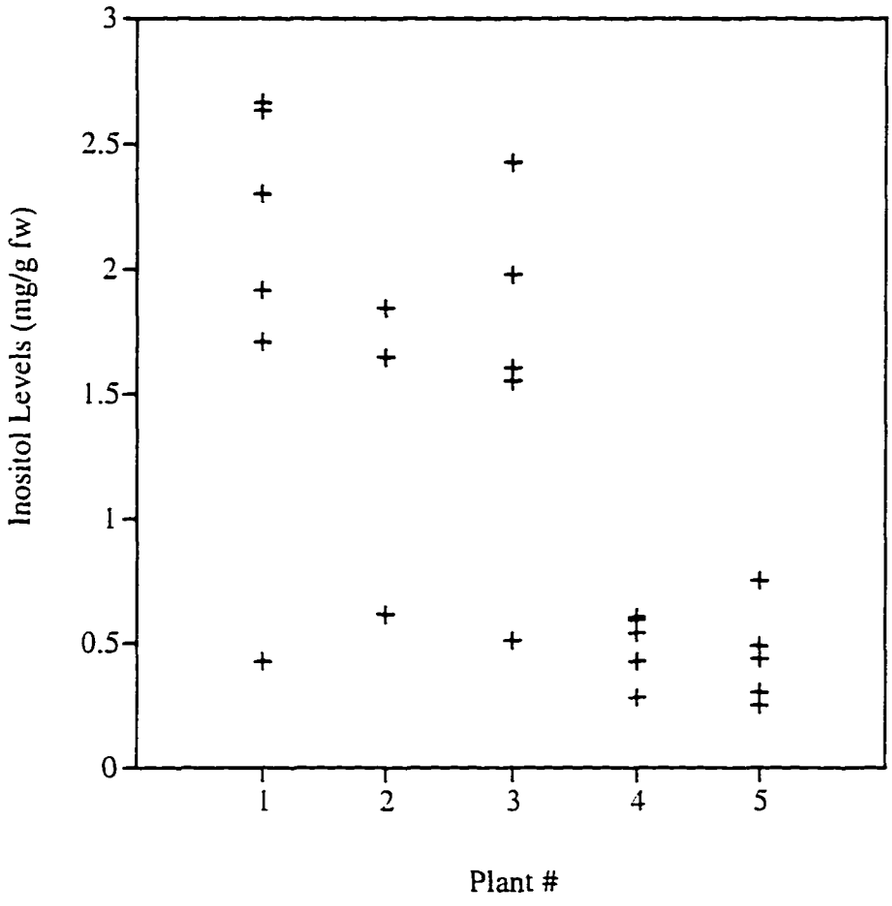


Fig. 6.2C Fructose levels in leaves from 5 young tomato plants

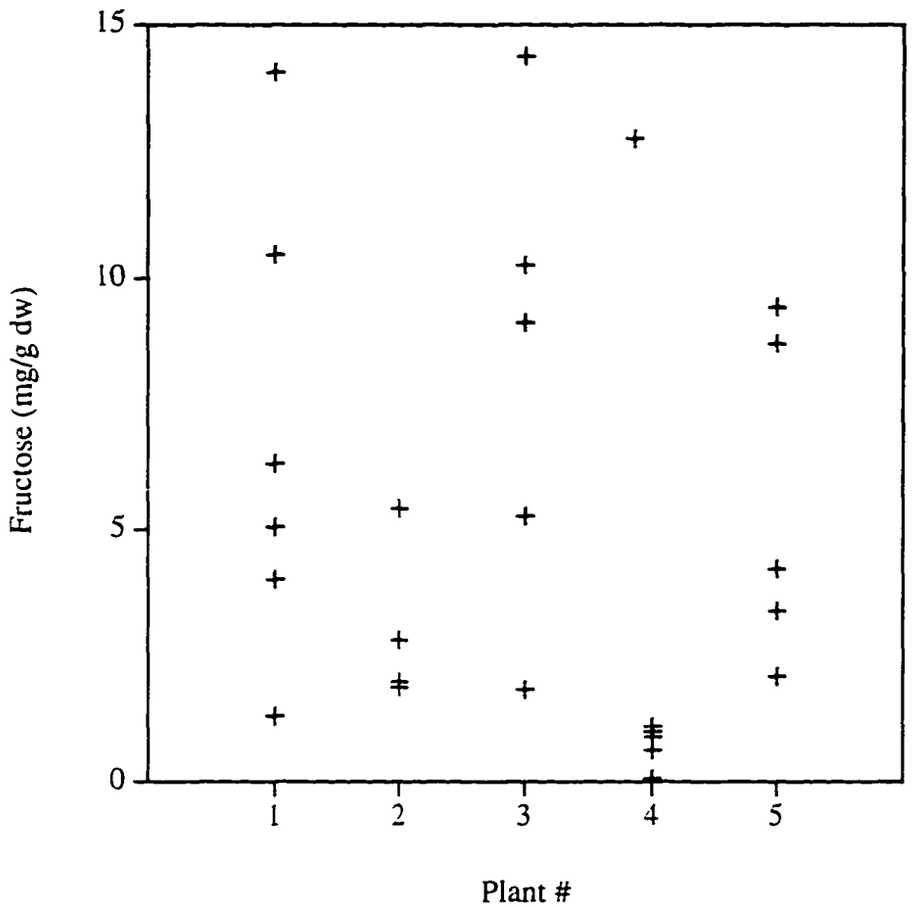


Fig. 6.2D Glucose levels in leaves from 5 young tomato plants

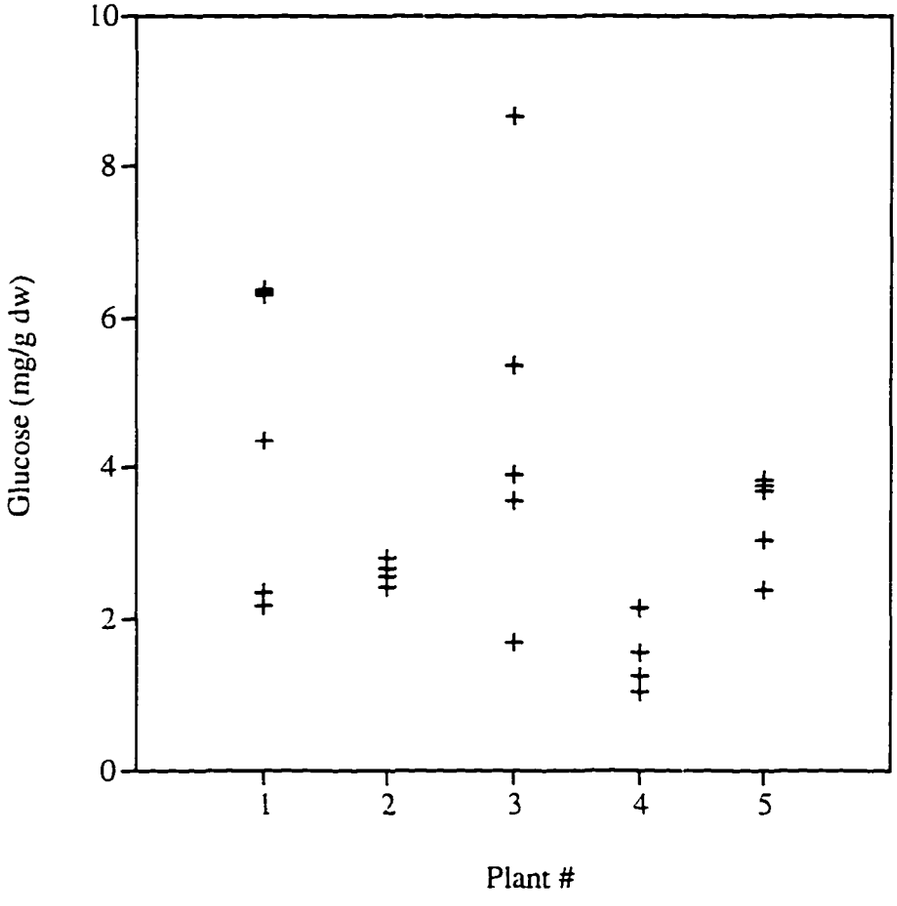


Fig. 6.2E Sucrose levels in leaves from 5 young tomato plants

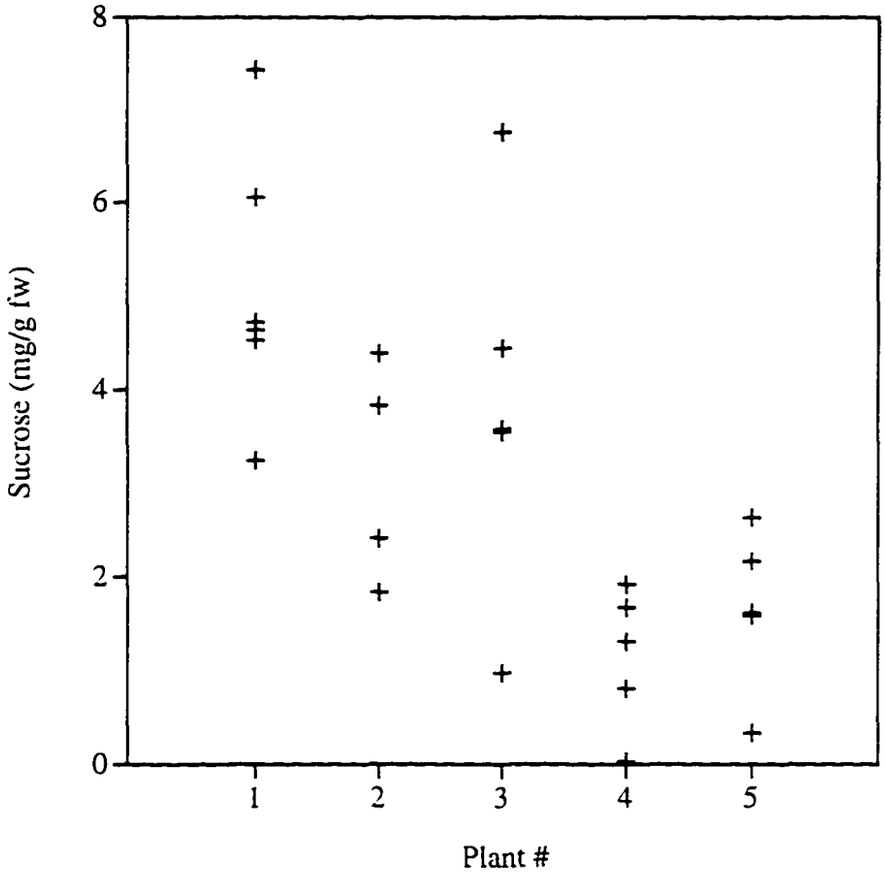


Fig. 6.3A Protein levels in leaves from young tobacco plants

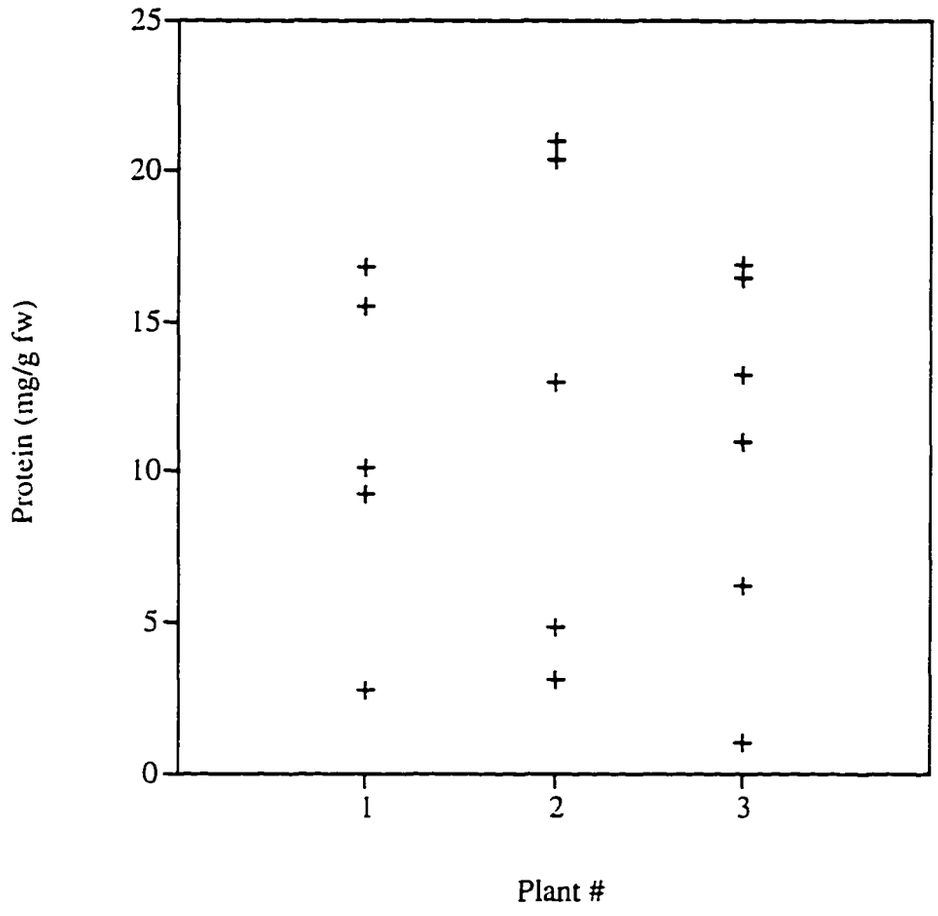


Fig 6.3B Inositol levels in leaves from young tobacco plants

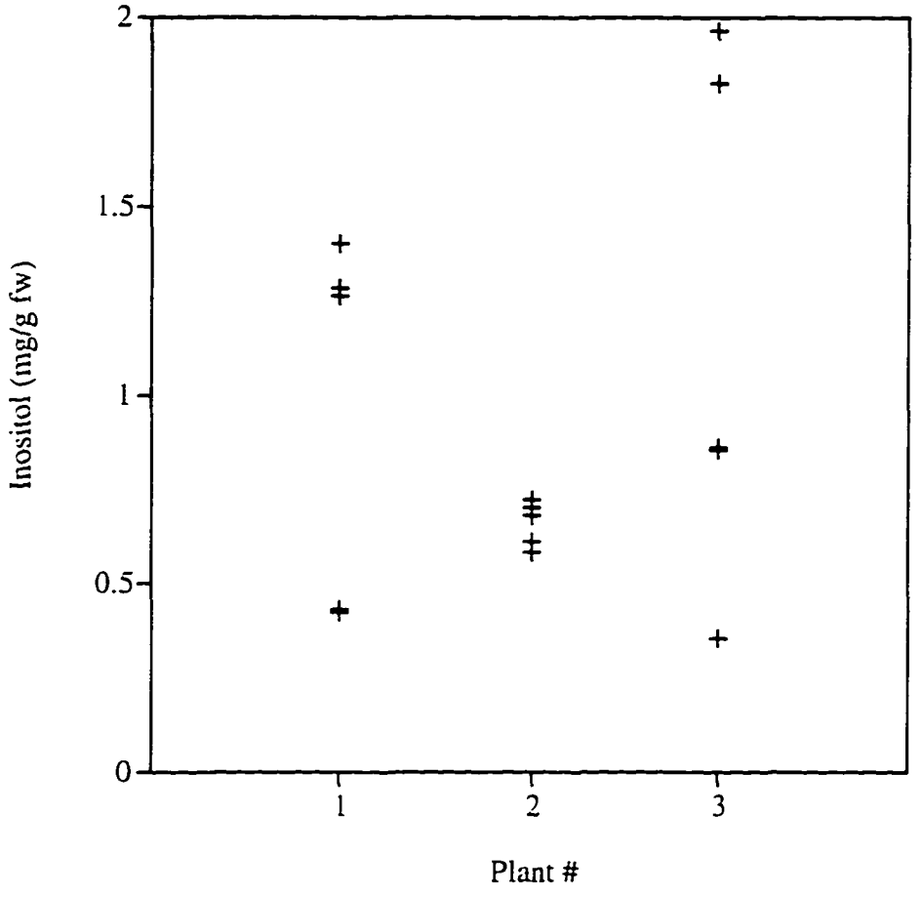


Fig 6.4A Protein levels in the tomato plants used in series 2.

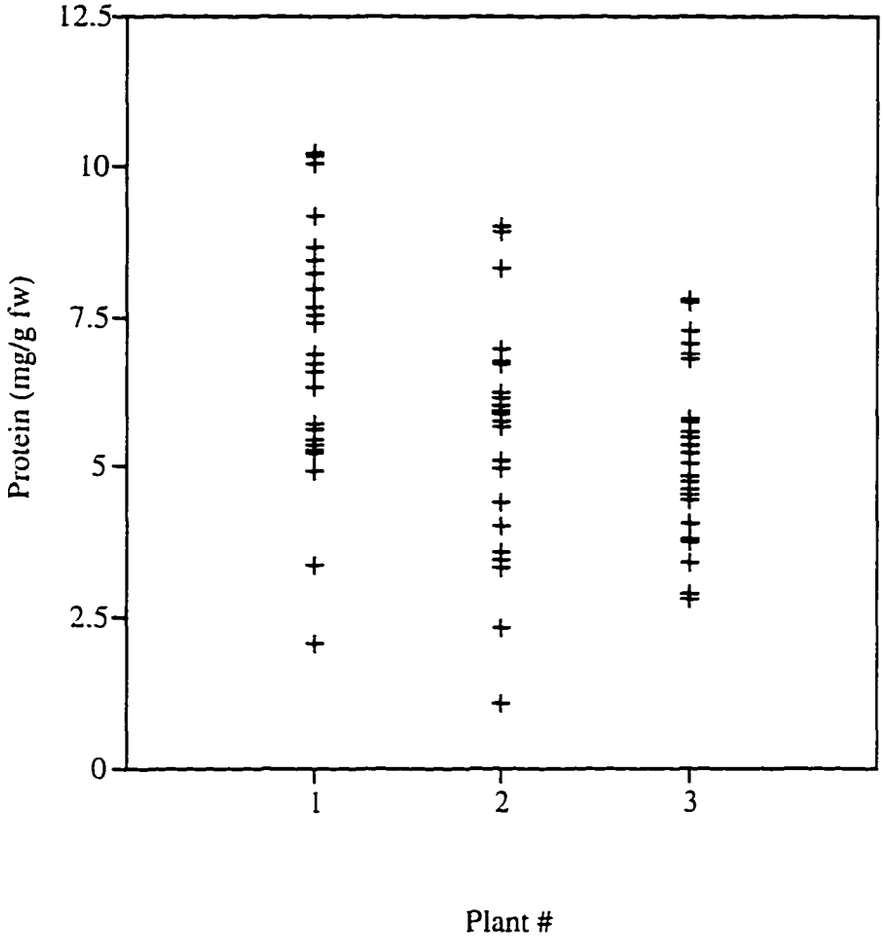


Fig 6.4B Inositol levels on the leaflet surface and within the tissue of the tomato plants analyzed in series 2.

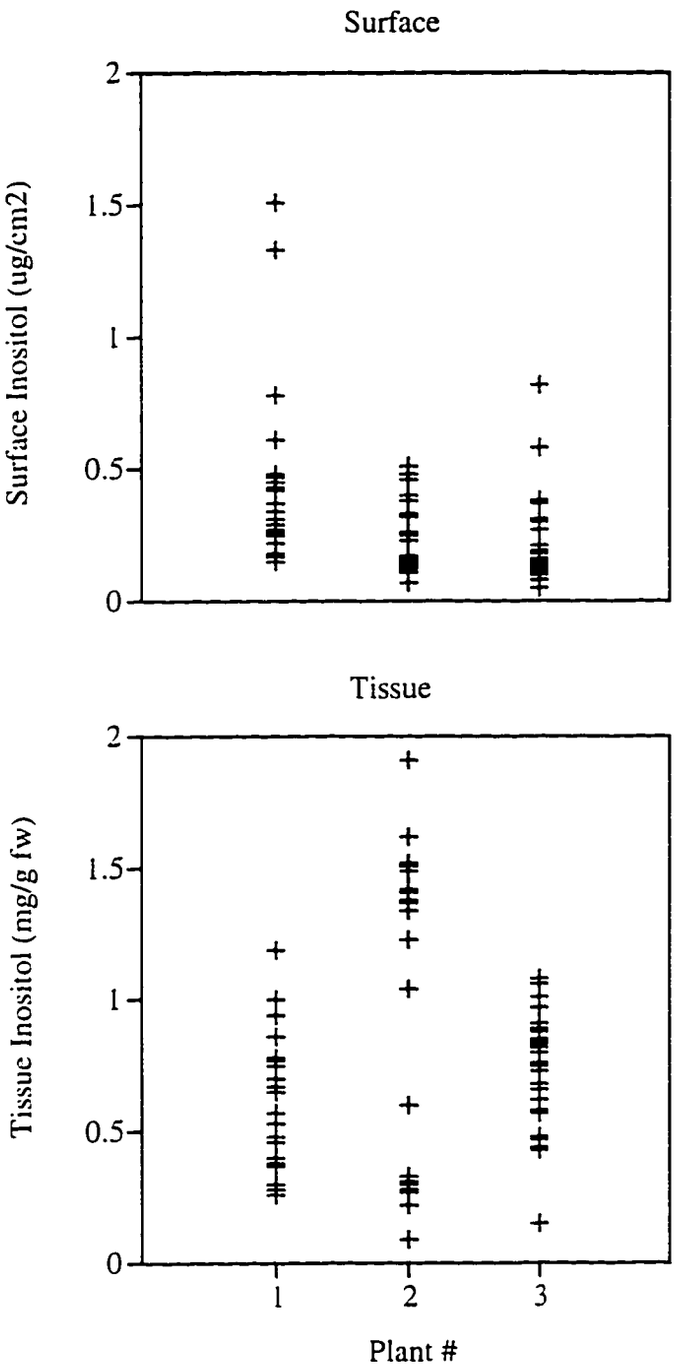


Fig 6.4C Fructose levels on the leaflet surface and within the tissue of the tomato plants analyzed in series 2.

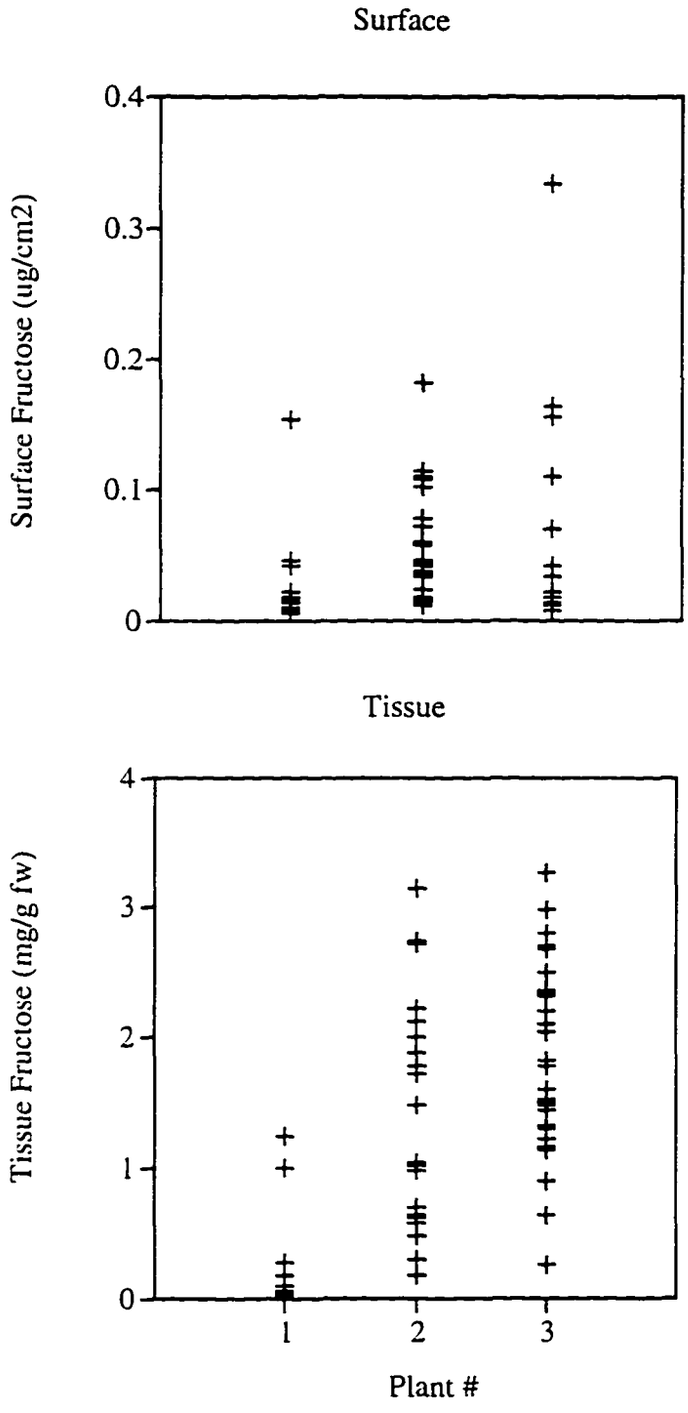


Fig 6.4D Glucose levels on the leaflet surface and within the tissue of the tomato plants analyzed in series 2.

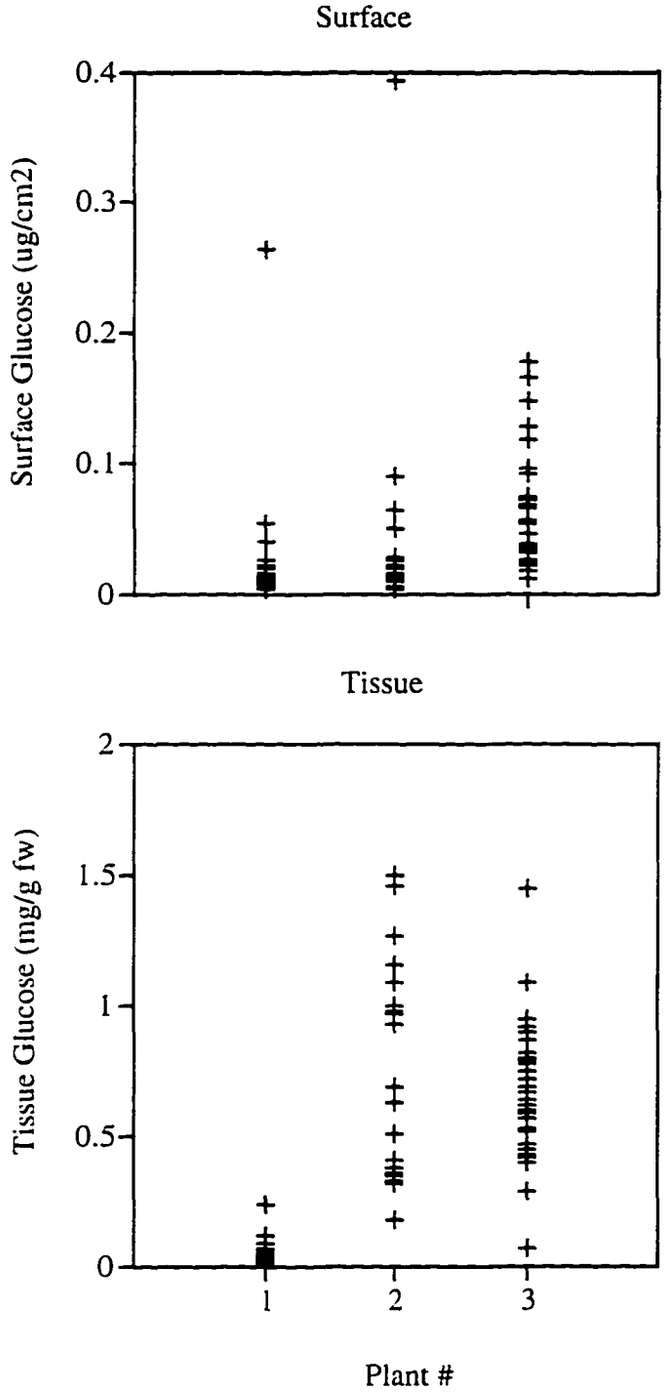


Fig 6.4E Sucrose levels on the leaflet surface and within the tissue of the tomato plants analyzed in series 2.

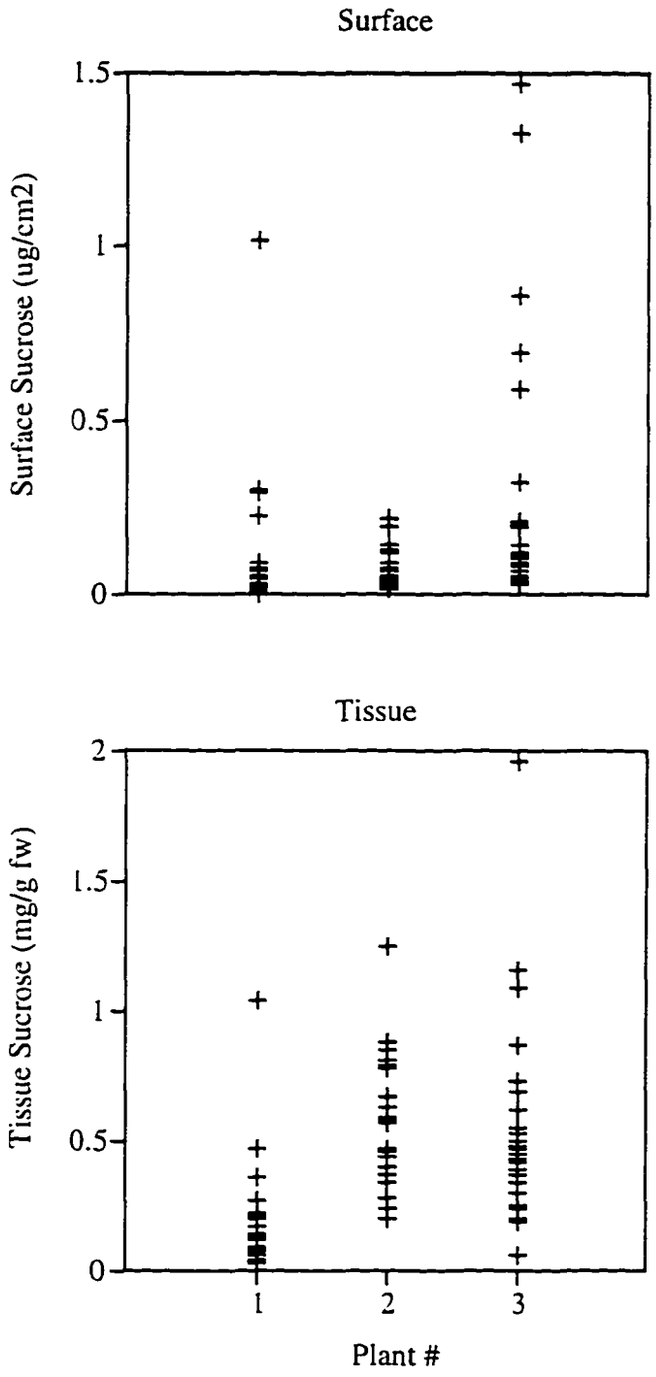


Fig. 6.5A Protein levels in the leaves from the tobacco plants used in series 2.

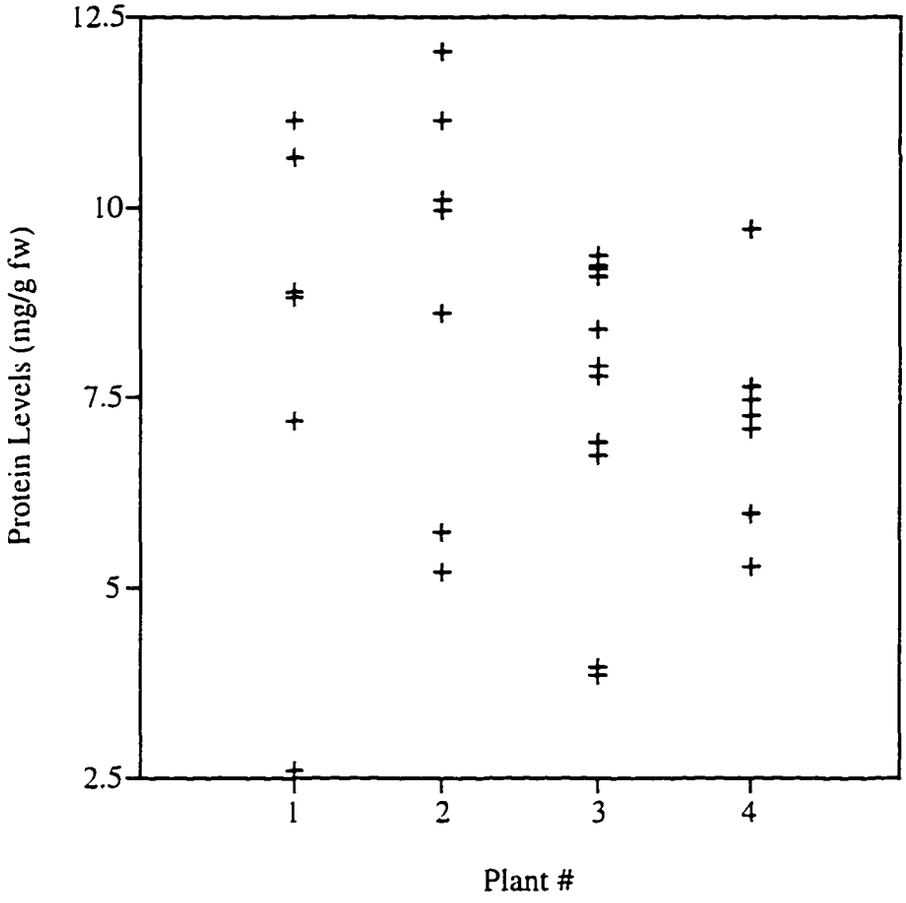


Fig 6.5B Inositol levels on the leaf surface and within the tissue of the tobacco plants analyzed in series 2.

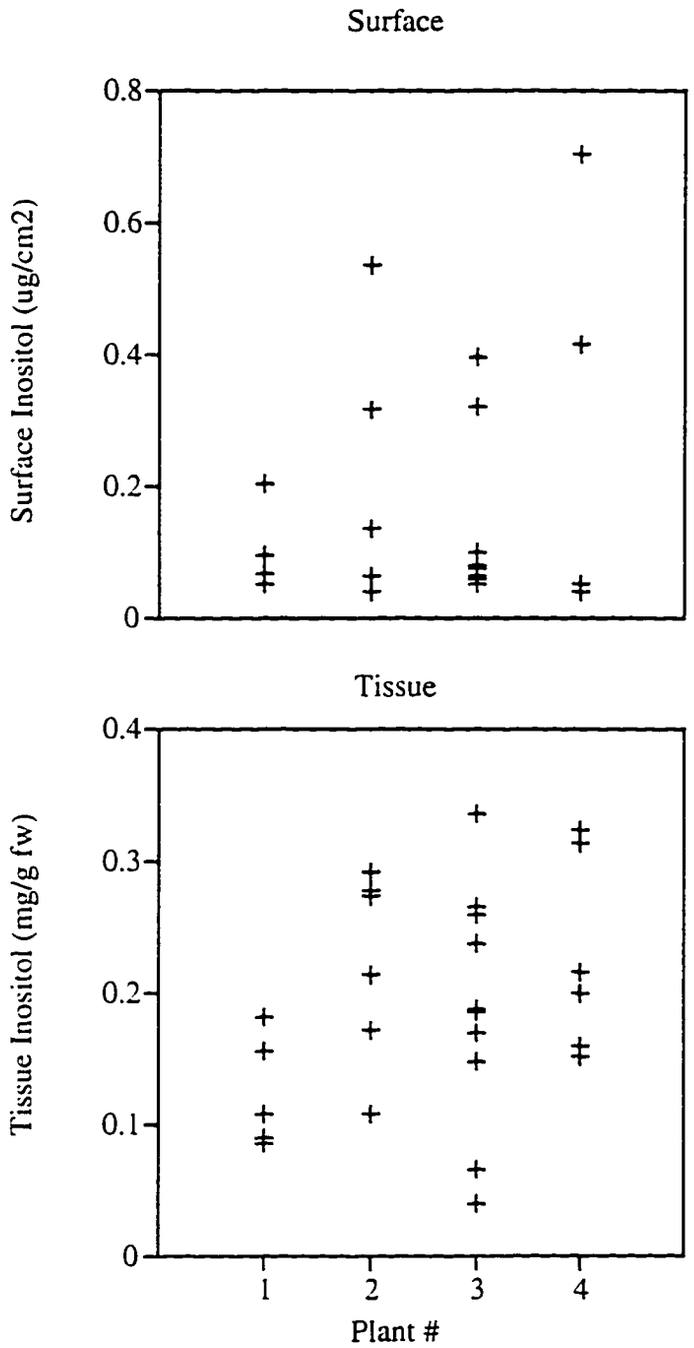


Fig 6.5C Sucrose levels on the leaf surface and within the tissue of the tobacco plants analyzed in series 2.

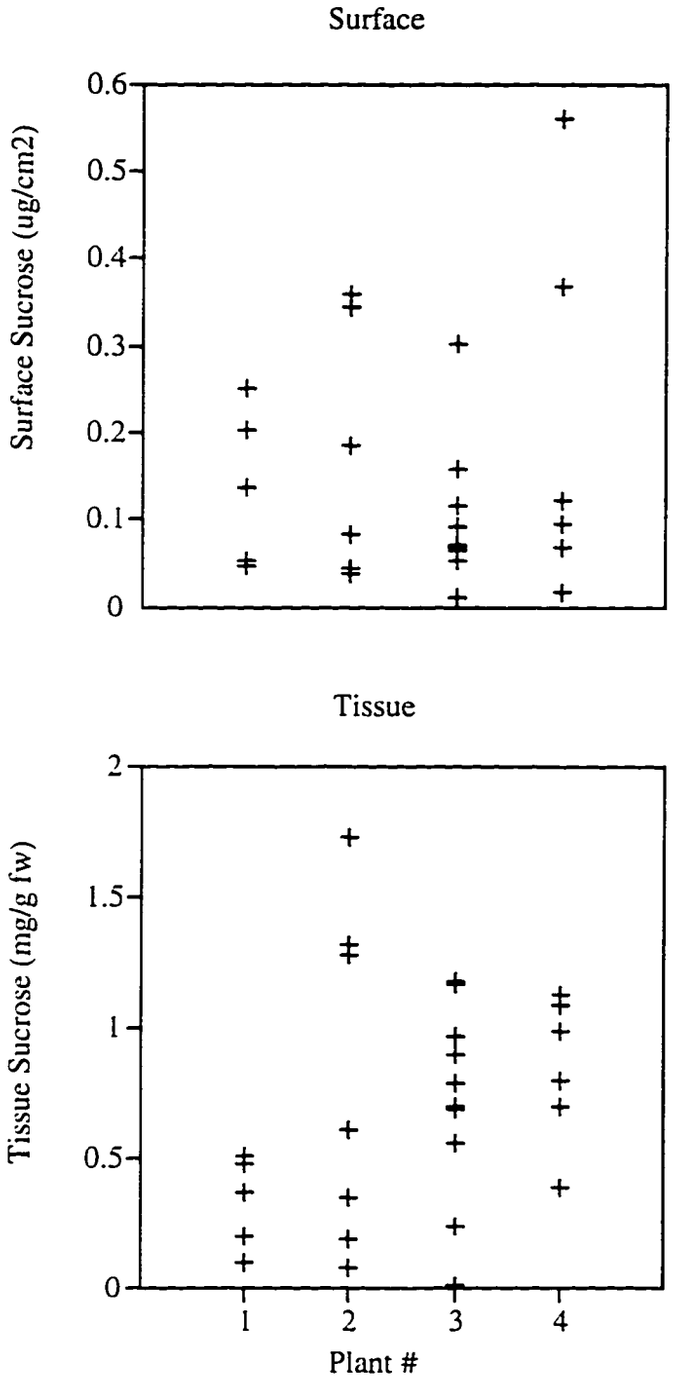


Fig 6.5D Glucose levels on the leaf surface and within the tissue of the tobacco plants analyzed in series 2.

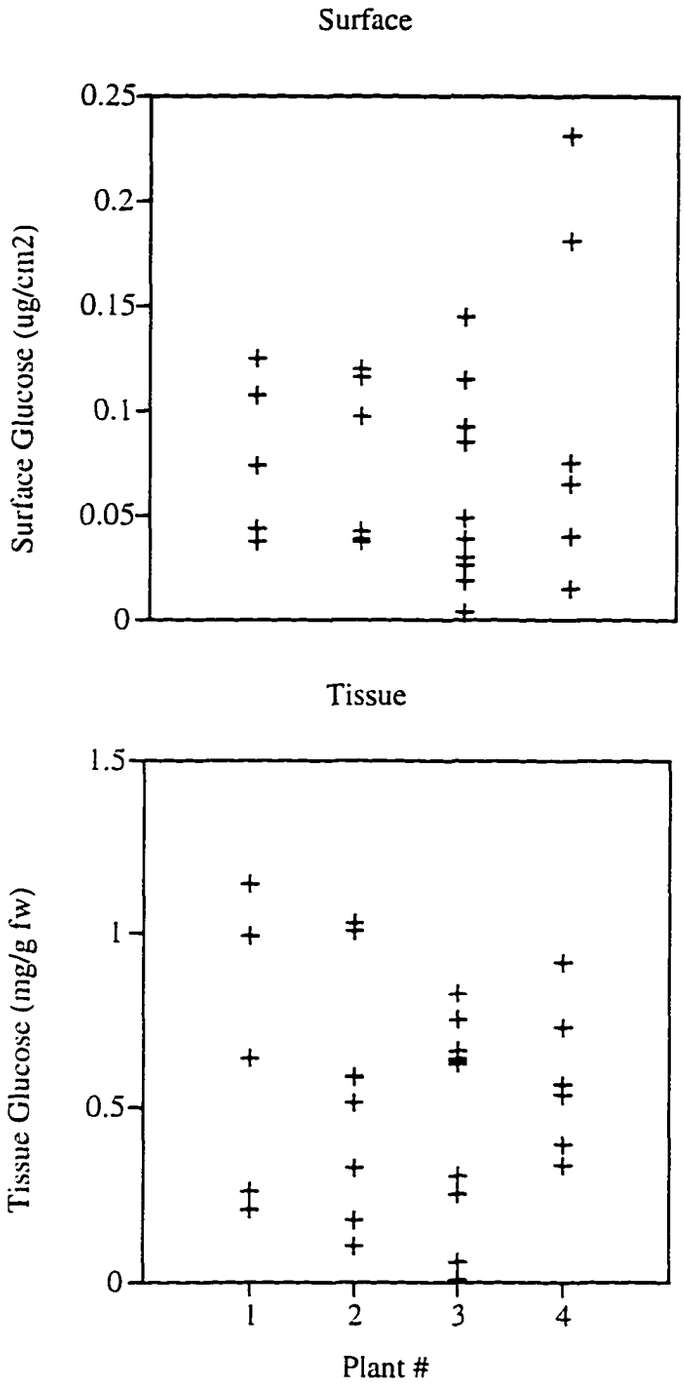
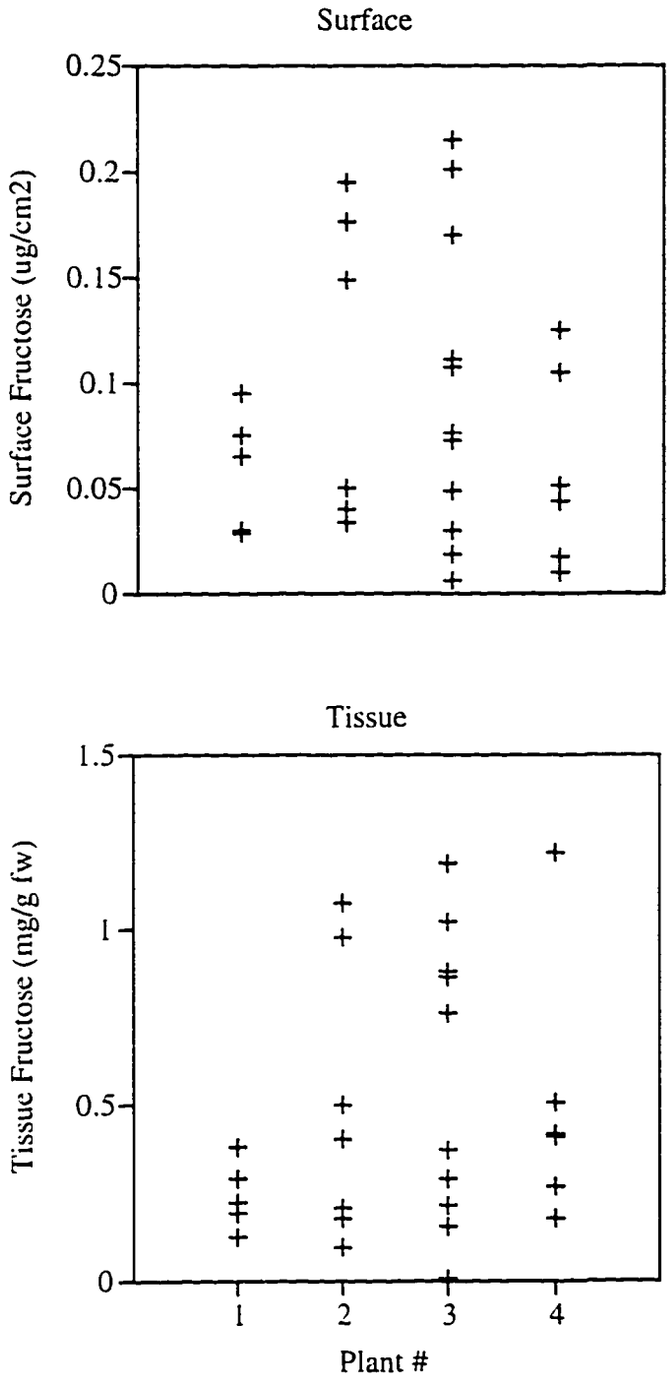


Fig 6.5E Fructose levels on the leaf surface and within the tissue of the tobacco plants analyzed in series 2.



CHAPTER 7: GENERAL CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

This study began as an investigation into lepidopteran taste receptors' sensitivity to *myo*-inositol. Electrophysiology confirmed that the species under study, *Manduca sexta*, has sensitivity to inositol in both the lateral and medial styloconica sensilla. Furthermore, a wide range of nutrients and similar compounds was tested at appropriate concentrations, and only one other compound, *epi*-inositol, elicited responses that were relatively high. However, *epi*-inositol only occurs in trace amounts in plants, so that sensitivity to *myo*-inositol remains the ecologically relevant response. An interesting and perhaps important detail is that no other nutrient tested elicited responses comparable with that obtained from *myo*-inositol. Thus, the response by this insect to *myo*-inositol has here been shown to be extremely specific. Although this is commonly assumed in the literature, further work is needed to conclusively show that there is an inositol specific cell in each sensillum.

Inositol stimulates feeding to some extent in various lepidopterans including *M.sexta*, but the data presented here show that amount eaten does not reliably indicate it is a strong phagostimulant. On many occasions, filter paper with water only was eaten at least as much as filter paper with inositol solutions. However, an interesting additional finding was that inositol appears to have a role in initiating feeding as opposed to causing increased quantities eaten, and in some cases positive overall effects of inositol as a phagostimulant could be accounted for by this effect alone. Moreover, in comparison to the sugars, the levels of inositol on leaf surfaces were proportionally much higher levels within the tissue. Thus, larvae may be using the high levels of inositol on the leaf surface as a general plant cue.

There is still work to be done to understand why the electrophysiological response to inositol is so vigorous, while the effect on behavior is not sustained during the course of

feeding. Inositol may play a more important role in feeding when it occurs in mixtures with other plant components. This is suggested by the work of Schoonhoven (1969) showing that the addition of inositol to an unacceptable non-host of *M.sexta* caused it to become acceptable.

The results of Chapter 4 indicate that, in contrast to some other species, inositol is not an essential dietary nutrient for *M.sexta* larvae, though required for egg production. This may be a quantitative effect if the insects are able to make enough for their own cell membranes but not enough for their eggs. Future work would first require the development of a suitable, defined diet that is totally synthetic so that trace amounts of dietary inositol in phospholipids would not interfere with an accurate measure of needs. Experiments to determine the amount of dietary inositol and at what stages of development dietary inositol is necessary for egg development are also needed. Effects of dietary inositol on the male reproductive system should also be investigated.

Chapter 4 also documents the ability of *M.sexta* larvae to utilize inositol as an energy source. This is unusual and may be related to the fact that the hosts examined, tobacco and tomato, had relatively low levels of sugars and relatively high levels of inositol. Thus there may be a direct value in detecting inositol in plant in that it can be used as major nutrient. Since these insects can use inositol as an energy source and because in some Solanaceous plants inositol levels are comparable to the levels of some of the sugars, selection may have favored larvae with taste receptors that were specific for inositol. Further studies on the relative utilization efficiency of inositol and other carbohydrates are needed.

Comparative studies among the Lepidoptera would be valuable to examine the stimulating effectiveness and nutritional uses of inositol in relation to host plant characteristics. The literature survey indicated that generalists were more commonly stimulated by inositol than were specialists; this needs confirmation. If found to be the

rule, its significance requires additional study. It is possible that generalists use inositol as a general plant cue, whereas specialist depend more on host-specific cues.

With respect to oviposition, moths laid more eggs on leaves with higher protein levels, but this was related to precontact cues. The surface compounds studied did not appear to influence post-alighting choice nor did they provide information with respect to tissue protein levels. However, other unknown post-alighting cues did appear to influence oviposition choice.

Two host plant species were examined for levels of protein, sugars and inositol. There was a significant positive relationship between tissue levels of protein and inositol in the young plants tested but not in the older plants. Since the larvae probably cannot taste protein directly and protein is the most critical major nutrient, this relationship between protein and inositol in younger plants may be of value to them; inositol can be a cue for the protein. The lack of relationship between surface inositol and tissue protein certainly means that inositol is not a particularly useful cue for adults, even if they can detect it. Further work is needed to clarify the correlation between inositol and protein, and to examine a range of different host plant species. If the positive correlation were found to be prevalent, it would stimulate new interest into the area of inositol sensitivity in lepidopterans and why this may vary.

REFERENCES

Abisgold, J.D. and S. J. Simpson. 1987. The physiology of compensation by locusts for changes in dietary protein. J. exp. Biol. 129: 329-346.

Abisgold, J.D. and S.J. Simpson. 1988. The effect of dietary protein levels and haemolymph composition on the sensitivity of the maxillary palp chemorecptors of locusts. J. exp. Biol. 135: 215-229

Adams, P., A. Zegeer, H.J. Bohnert, and R.G. Jensen. 1993. Anion exchange separation and pulsed amperometric detection of inositols from flower petals. Anal. Bioch. 214:321-324.

Ahmad, S. 1983. Herbivorous Insects: Host-Seeking Behavior and Mechanisms. Academic Press. New York.

Baldwin, I.T. 1988. The alkaloidal responses of wild tobacco to real and simulated herbivory. Oecologia 77:378-.

Baldwin, I.T. and T.E. Ohnmeiss. 1994. Coordination of photosynthetic and alkaloidal responses to damage in uniducible and inducible *Nicotiana sylvestris*. Ecology 75(4):1003-1014.

Banderski, R.S., A. Schulze, J.D. Cohen. 1977. Photo-regulation of the ration of ester to free indole-3-acetic acid. Biochem Biophys Res Commun. 79:1219-1233.

Berdanier, C.D. 1992. Is inositol and essential nutrient. Nutr. Today 27(2):22-26.

Bernays, E.A. and R.F. Chapman. 1994. Host-Plant Selection by Phytophagous Insects. Chapman and Hall. New York, NY.

Bernays, E.A., J.J. Howard, D. Champagne, and B.J. Estesen. 1991. Rutin: a phagostimulant for the polyphagous acridid *Schistocerca americana*. Entomol. exp. appl. 60:19-28.

Berridge, M.J. 1983. Rapid accumulation of inositol trisphosphate reveals that agonist hydrolyse polyphyosphoinositides instead of phosphatidylinositol. Biochem. J. 212:849-858.

Billington, D.C. 1993. The Inositol Phosphates. Chemical Synthesis and Biological Significance. VCH. Weinheim.

Blaney, W.M. and M.S.J. Simmonds. 1988. Food selection in adults and larvae of three species of Lepidoptera: a behavioural and electrophysiological study. Entomol. exp. appl. 49:111-121.

Coté, G.G. and R.C. Crain. 1993. Biochemistry of phosphoinositides. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44:333-356.

Dadd, R.H. 1960. The nutritional requirements of locusts. I. Development of synthetic diets and lipid requirements. J. Insect Physiol. 11: 211-223.

Dadd, R.H. 1961. The nutritional requirements of locusts-IV. Requirements for vitamins of the B complex. J. Ins. Physiol. 6: 1-12.

Dadd, R.H. 1977. Qualitative requirements and utilization of nutrients: insects. In CRC Handbook Series in Nutrition and Food (M. Rechcid, ed.). Vol.1 Sec. D. Nutritional requirements. pp. 305-346

Dadd, R.H. 1984. Chapter 8, Nutrition: Organisms. In Comprehensive Insect Physiology, Biochemistry, and Pharmacology (G.A. Kerkut and L.I. Gilbert, eds.). Volume 4 Regulation: Digestion, Nutrition, Excretion. pp. 313-390.

Den Otter, C.J. 1992. Responses of the African armyworm and three species of borers to carbohydrates and phenolic substances: an electro-and behavioural physiological study. Entomol. exp. appl. 63: 27-37.

Derridj, S., V. Gregoire, J.P. Boutin, and V. Fiala. 1989. Plant growth stages in the interspecific oviposition preference of the European corn borer and relations with chemicals present on the leaf surface. Entomol. exp. appl. 53:267-276.

Dethier, V.G. 1973. Electrophysiological studies of gustation in lepidopterous larvae. II. Taste spectra in relation to food-plant discrimination. J. comp. Physiol. 82:103-134.

Dethier, V.G. and J.H. Kuch. 1971. Electrophysiological studies of gustation in lepidopterous larvae. I. Comparative sensitivity to sugars, amino acids, and glycosides. Z. vergl. Physiologic 72: 343-363.

Eigenbrode, S.D., K.A. Stoner, A.M. Shelton, W.G. Kain. 1991. Characteristics of glossy leaf waxes associated with resistance to diamondback moth (Lepidoptera: Plutellidae) in *Brassica oleracea*. Journal of Economic Entomology 84(5):1609-1617.

Everly, R.R. 1959. Influence of higth and stage of development of dent corn on oviposition by European Corn Borer moths. Annals of the Entomological Society of America 52:272-279.

Fernando-Warnakulasuriya, G.J.P. , K. Tsuchida, and M.A. Wells. 1988. Insect Biochem. 18(2):211-214.

Forgash, A.J. 1958. The effects of inositol on growth, survival and maturation in *Periplaneta americana* (L.). Ann. Entomol. Soc. Amer. 51: 406-409.

Gilroy, S., N.D. Read, and A.J. Trewavas. 1990. Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stromatal closure. Nature346:769-771.

Gordon, H.T. 1959. Minimal nutrition requirements of the German cockroach, *Blattella germanica* L. Ann. N.Y. Acad. Sci. 77:290-351.

Gothilf, S. and F.E. Hanson. 1994. A technique for electrophysiologically recording from chemosensory organs of intact caterpillars. Entomol. exp. appl. 72:305-310.

Guenther, A.B., R.K. Monson, R. Fall. 1991. Isoprene and monoterpene emission rate variability: observations with Eucalyptus and emission rate algorithm development. Journal of Geophysical Research 96(D6):10799-10808.

Hanson, F.E., S. Kogge, C. Cearley. 1985. Computer analysis of chemosensory signals. In Mechanisms in insect olfaction. T.L. Payne, M.C. Birch, and C.E.J. eds. Clarendon Press. Oxford. p, 269-278.

Helsper, J.P.F.G., P.M.F. DeGroot, H.F. Linkens, and J.F. Jackson. 1986. Phosphatidylinositol monophosphate in *Lilium* pollen and turnover of phospholipid during pollen tube extension. Phytochemistry 25:2193-2199.

Hodges, Ronald W. 1971. The Moths of America North of Mexico. Spingoide. Fascicle 21. E.W. Classey Limited and RBD Publications Inc. London.

Horie, Y., K. Watanabe, and T. Ito. 1966. Nutrition of the silkworm, *Bombyx mori* XIV. Further studies on the requirements for B vitamins. Bull. Sericul. Exp. Sta., 20(4): 393-409.

Hsiao, T.H. 1985. Feeding behavior. In: G.A. Kerkut and L.I. Gilbert (eds.) Comprehensive Insect Physiology, Biochemistry, and Parmacology. Vol. 9. Pergamon Press, Oxford, pp. 471-512.

Ishikawa, S., T. Hirao, and N. Arai. 1969. Chemosensory basis of hostplant selection in the silkworm. Ent. exp & appl. 12:544-554.

Jones, C. G., J.D. Hare, and S. Compton. 1989. Measuring plant protein with the Bradford assay. J. Chem. Ecol. 15(3):979-992.

Journet, A.R.P. 1980. Intraspecific variation in food plant favourability to phytophagous insects: psyllids on *Eucalytus blakelyi* M. Ecological Entomology 5:249-261.

Jung, P., W. Tanner, and K. Wolter. 1972. The fate of *myo*-inositol in *Fraxinus* tissue culture. Phytochemistry 11:1655-1659.

Loewus, F.A. 1990. Stucture and occurrence of inositols in plants. In F.A. Loewus (ed.) "Inositol Metabolism in Plants," Wiley-Liss, Inc., pp.1-11.

Loewus, F.A., J.D. Everard, and K.A. Young. 1990. Inositol metabolism: precursor role and breakdown. In F.A. Loewus (ed.) "Inositol Metabolism in Plants," Wiley-Liss, Inc., pp. 21-45.

Ma, W.C. 1972. Dynamics of feeding responses in *Pieris brassicae* Linn. as a function of chemosensory input: a behavioural, ultrastructural and electrophysiological study. Meded. Landbouwhogeschool Wageningen 72-11(1972).

Madden, A.H. 1945. Biology of the tobacco hornworm in the southern cigar-tobacco district. USDA Technical Bulletin No. 896. 51p.

McFadden, M.W. 1968. Observations on feeding and movement of tobacco hornworm larvae. Journal of Economic Entomology 61(2):352-356.

Myers, J.H. 1985. Effects of physiological condition of the host plant on the ovipositional choice of the cabbage white butterfly, *Pieris rapae*. Journal of Animal Ecology 54:193-204,

Michalczuk, L. R.G. Baldi, and J.D. Cohen. 1990. *myo*-Inositol conjugates of indole-3-acetic acid. In F.A. Loewus (ed.) "Inositol Metabolism in Plants," Wiley-Liss, Inc., pp. 93-106.

Moore, T.S. Jr. 1990. Biosynthesis of phosphatidylinositol. In F.A. Loewus (ed.) "Inositol Metabolism in Plants," Wiley-Liss, Inc., pp. 107-112.

Nielsen, C.S. and I.F.B. Common. 1991. Chapter 41 Lepidoptera. In: "The Insects of Australia. A Textbook for Students and Research Workers. Volume II." Cornell University Press.

Ozalp, P. and I. Emre. 1992. Effects of water-soluble vitamins on egg production and hatchability of adult *Pimpla turionellae*. Turkish Journal of Zoology 16(1): 78-83.

Papaj, D.R. and M.D. Rausher. 1987. Components of conspecific host discrimination behavior in the butterfly *Battus philenor*. Ecology 68(2):245-253.

Ramaswamy, S.B. (ed.) 1988. Host finding and feeding in adult phytophagous insects. J. Insect Physiol. 34:115-268.

Rees, C.J.C. 1969. Chemoreceptor specificity associated with the choice of feeding site by the beetle, *Chrysolina brunsvicensis* on its foodplant, *Hypericum hirsutum*. Entomol. exp. appl. 12:565-583.

Ryan, C.A. 1987. Oligosaccharide signalling in plants.Annu Rev Cell Biol 3:295-317.

Sacher, R.F. and R.C. Staples. 1985. Inositol and sugars in adaptations of tomato to salt. Plant Physiol. 77:206-210.

Schoonhoven, L.M. 1969a. Sensitivity changes in some insect chemoreceptors and their effect on food selection behavior. In Koninkl. Nederl. Akademie Van Wetenschappen in Amsterdam, Proc., Series C. 72:491-498.

Schoonhoven, L.M. 1969b. Gustation and foodplant selection in some lepidopterous larvae. Entomologia. exp. appl. 12: 555-564.

Schoonhoven, L.M. 1972. Plant recognition by lepidopterous larvae. Proc. Symp. R. Ent. Soc., Lond. 6:87-99.

Schoonhoven, L.M. 1974. Comparative aspects of taste receptor specificity. In: Transduction Mechanisms in Chemoreception. Proceedings of the European Chemoreception Research Organization. Surrey, England. T.M. Poynder ed. 198-201.

Schoonhoven, L.M. 1976. On the variability of chemosensory information. Symp. biol. Hung. 16:261-266.

Schoonhoven, L.M. 1987. What makes a caterpillar eat? The sensory code underlying feeding behavior. In: Perspectives in Chemoreception and Behavior. E.A. Bernays, R. Chapman, and J.G. Stoffolano Jr. eds. pp. 69-97.

Simmonds, M.S.J., L.M. Schoonhoven, and W.M. Blaney. 1991. Daily changes in the responsiveness of taste receptors correlate with feeding behaviour in larvae of *Spodoptera littoralis.* Entomologia. exp. appl.

Scriber, J.M. and F. Slansky. 1981. The nutritional ecology of immature insects. Annu. Rev. Entomol. 26:183-211.

Scriber, J.M. and F. Slansky. 1985. Food consumption and utilization. In Comprehensive Insect Physiology, Biochemistry and Pharmocology. Vol. 4 Regulation: Digestion, Nutrition, and Excretion. G.A. Kurkut and L.I. Gilbert. eds. Pergamon Press. Elmsford, NY.

Simpson, S.J. 1990. The pattern of feeding. In "Biology of Grasshoppers" Chapter 3. R.F. Chapman and A. Joern eds. John Wiley and Sons, New York.

Smith, J.J.B., B.K. Mitchell, B.M. Rolseth, A.T. Whitehead, and P.J. Albert. 1990. SAPID tools: Microcomputer programs for analysis of multi-unit recordings. Chem. Senses 15:253-270.

Städler, E. and F.E. Hanson. 1976. Influence of induction of host preference of chemoreception of *Manduca sexta*: Behavioral and electrophysiological studies. Symp. Biol. Hung. 16:267-273.

Städler, E. and F.E. Hanson. 1978. Food discrimination and induction of preference for artificial diets in the tobacco hornworm, *Manduca sexta*. Phys. ent. 3:121-133.

Tukey, H.B. 1971. Leaching of substances from plants. In: Ecology of Leaf Surface Micro-Organisms. T.F. Preece and C.H. Dickinson eds.. Academic Press. NY. pp. 67-80.

Turunen, S. 1989. Absorption and metabolism of dietary *myo*-inositol in *Pieris brassicae*. Insect Biochem. 19(6):603-610.

van Drongelen, W. and G.D.E. Povel. 1980. Gustatory sensitivity and taxonomic relationships in larvae of some *Yponomeuta* species (Lepidoptera). Proc. Kon. Ned. Akad. Wet.

Vanderzant, E.S. 1959. Inositol: an indispensable dietary requirement for the boll weevil. Journal of Economic Entomology. 52(5):1018-1019.

Vanderzant, E.S. 1968. Dietary requirements of the bollworm, *Heliothis zea* (Lepidoptera: Noctuidae), for lipids, choline, and inositol and the effect of fats and fatty acids on the composition of the body fat. Annals of the Entomological Society of America. 61(1): 120-125.

Waldbauer, G.P. and S. Friedman. 1991. Self-selection of optimal diets by insects. Annu Rev Entomol. 36: 43-63.

Wolter K.E., and L. Murmanis. 1977. Radioautography of *myo*-inositol in cultured *Fraxinus* callus. New Phytol. 78:95-99.

Yamamoto, R.T. and G. Fraenkel. 1960. Assay of the principal gustatory stimulant for the tobacco hornworm, *Protoparce sexta*, from Solanaceous plants. Ann. Ent. Soc. Am. 53:499-507.

Zobel, A.M. and S.A. Brown. 1988. Determination of furanocoumarins on the leaf surface of *Ruta graveolens* with an improved extraction technique. Journal of Natural Products 51(5):941-946.