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Alkaloid tolerance in *Manduca sexta* and phylogenetically related sphingids (Lepidoptera: Sphingidae)

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Summary. Nicotine tolerance is well known for *Manduca sexta*. It also occurs in several other sphingids of the subfamilies Macroglossinae and Sphinginae. Only members of the subfamily Smerinthinae appear to be more susceptible to nicotine intoxication. Phylogenetic trees have been reconstructed from mitochondrial 16S rDNA and nuclear DNA to map nicotine tolerance.

The nicotine binding site of both α-subunits of nicotinic acetylcholine receptors (nAChR) have been amplified and sequenced. No apparent amino acid substitution can be seen in the putative nicotine binding site of the α-subunits of nAChR from nicotine tolerant and nicotine sensitive sphingids. Thus, a simple target-site modification can be ruled out as a cause for nicotine tolerance. This finding agrees with feeding experiments: larvae of *M. sexta* and other sphingids of the Macroglossinae and Sphinginae not only tolerated nicotine, but also many other alkaloids that affect neuroreceptors other than acetylcholine receptors (nAChR, mAChR).

Only 10 to 20% of nicotine injected into larvae of nicotine-tolerant taxa could be recovered later as free nicotine, nicotine N-oxide or cotinine, *i.e.*, 80 to 90% must have been converted to polar conjugates or degradation products which are not detectable with the methods applied. Usually more than 98% of the recoverable alkaloids were found in the faeces. Excretion reached a maximum 6 h after injection in tolerant taxa. Larvae of *Manduca sexta*, which were reared on a nicotine-rich diet, showed higher nicotine degradation and faster nicotine elimination than naïve larvae. Application of the cytochrome *P*450 inhibitor SKF 525A (proadifen) reduced the formation of nicotine N-oxide and the rate of alkaloid degradation. Thus, an inducible detoxification mechanism, coupled with a rapid and inducible excretion, appear to be a strategy in Sphingidae that helps them to live on host plants rich in otherwise toxic secondary metabolites.

Key words. *Manduca sexta* – Sphingidae – nicotine – alkaloids – nicotinic acetylcholine receptor – 16S rRNA phylogeny – alkaloid metabolism – alkaloid tolerance

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Introduction

The pyridine alkaloid nicotine is a major alkaloid of tobacco (Solanaceae: *Nicotiana* spp.) and occurs as a minor component in several other non-solanaceous plants.

Nicotine is a strong agonist at nicotinic acetylcholine receptors (nAChR) in animals, and has been used as an efficient insecticide (Wink 1993a). Only few insects are known, that can tolerate nicotine in their diet. The best studied example is the Tobacco hornworm (*Manduca sexta*), which is almost insensitive to nicotine. In contrast, for example, to butterflies and moths of the families Nymphalidae and Arctiidae, which store dietary cardiac glycosides or pyrrolizidine alkaloids, respectively (Wink 1993b; Bernays & Chapman 1994; Brown & Trigo 1995; Hartmann & Witte 1995) *Manduca sexta* does not store nicotine for chemical defence (Snyder *et al.* 1994; this study). Other members of the Sphingidae (representing members of the three subfamilies Macroglossinae, Sphinginae and Smerenthinae (Minet 1994)) exploit host plants that produce other toxic secondary metabolites; we are not aware of a report showing a storage of these compounds.

In this study we analysed whether nicotine tolerance is restricted to *Manduca sexta* or present also in other members of the Sphingidae. Experimental evidence suggests that a high alkaloid detoxification capacity and rapid alkaloid excretion are among the biochemical mechanisms that mediate nicotine insensitivity in *Manduca sexta* (Self *et al.* 1964a,b; Maddrell & Gardiner 1976; Snyder *et al.* 1993, 1994; Murray *et al.* 1994). We injected defined alkaloid solutions into the larvae of *Manduca sexta*, other Sphingidae and other Lepidoptera to determine their potential toxicity, and the degree and speed of alkaloid elimination.

Another possibility to explain nicotine tolerance would be a modification of the acetylcholine binding site of neuronal acetylcholine receptors (nAChR) analogous to that reported in the Monarch, *Danaus plexippus*. The Monarch and other Danainae take up cardiac glycosides (CG) from their host plants and exploit them as defence substances against predators

(Brower 1984). Cardiac glycosides, such as ouabain or calotoxin (which occurs in their *Asclepias* host plants), are toxic to animals not adapted to these compounds. The molecular target of cardiac glycosides is the Na^+, K^+-ATPase. This enzyme can not be inhibited by CGs: it is insensitive in the Monarch, though sensitive in other insects that do not sequester cardiac glycosides (Vaughan & Jungreis 1977). The first extracellular domain of the α-subunit of the Na^+, K^+-ATPase has been regarded as a binding site for ouabain (Price & Lingrel 1988). A few years ago, we amplified and cloned the DNA-sequence encoding the respective ouabain binding site. The putative ouabain binding site consists of 12 amino acids (position 111–122). In ouabain-sensitive insects asparagine occurs at position 122. The binding site of the Monarch shows a point mutation with a histidine instead at amino acid position 122 (Holzinger *et al.* 1992). Starting with the CG-sensitive Na^+, K^+-ATPase gene from *Drosophila*, we converted position 122 to a histidine residue as in *Danaus plexippus* by site-directed mutagenesis (Holzinger & Wink, 1996). Human embryonic kidney cells (HEK) (which are sensitive to ouabain) were transfected with the mutated Na^+, K^+-ATPase gene in a pSVDF-expression vector and showed a transient expression of the mutated *Drosophila* Na^+, K^+-ATPase. When treated with ouabain, the transfected cells tolerated ouabain at a concentration of 50 nM, whereas untransformed controls of controls transfected with the unmutated *Drosophila* gene showed a substantial cell death. This result implies that the asparagine to histidine exchange contributes to ouabain insensitivity in the Monarch. With respect to these results we analysed in this study whether the remarkable nicotine tolerance of *Manduca sexta* could be due to a modification of the acetylcholine binding site of the $\alpha 1$ of $\alpha 2$ subunits of the nicotinic AChR which bind nicotine with high affinity (Ohana & Gershoni 1990).

In a second part of this study we have reconstructed a molecular phylogeny of 17 sphingids that were included in the nicotine experiments using sequence data from a mitochondrial marker gene, 16S rDNA (DeSalle 1992; Pashley & Ke 1992; Wink & von Nickisch-Rosenegk 1997; Wink *et al.* 1998). The molecular phylogeny was used as a framework to infer the evolution of nicotine tolerance in *Manduca* and other sphingids.

Material and methods

Animals

The origins of insects and their laboratory culture conditions are summarised in Table 1. Most larvae were offered leaves of their respective host plants *ad libitum*. *Manduca sexta* was reared on an artificial diet according to Bergomaz & Boppré (1986). Larvae were kept individually in plastic cages (40 × 32 × 10 cm) or plastic beakers (diameter 10 cm; height 8 cm). Details of culture conditions are documented in Theile (1998). Larvae or eggs were obtained from several entomologists who breed these species in captivity.

Table 1 Origin of insects and their laboratory culture conditions

Species	Origin	Food plant
Sphingidae		
Acherontia atropos	Andalusia, Spain	*Solanum tuberosum*
Deilephila elpenor	Münster valley, Germany	*Epilobium* spp.
Hyles livornica	Belek, Turkey	*Epilobium* spp.; diet
Hyles euphorbiae	Syrgenstein, Germany	*Euphorbia cyparissias*
Hyles e. conspicua	Taurus mountains, Turkey	*Euphorbia cyparissias*
Manduca sexta	Laboratory culture	artificial diet
Sphinx ligustri	Weißenfels, Germany	*Ligustrum vulgare*
Sphinx pinastri	Aosta Valley, Switzerland	*Pinus sylvestris*
Smerinthus ocellatus	Birkholz, Germany	*Salix* spp. *Populus* spp.
Laothoe populi	Weißenfels, Germany	*Populus* spp.
Laothoe austauti	Nefza, Tunisia	unknown
Daphnis nerii	Patras, Greece	*Nerium oleander*
Mimas tiliae	Syrgenstein, Germany	*Tilia* spp.
Therectra alecto	Corfu, Greece	*Fuchsia* spp., *Epilobium* spp.
Nymphalidae		
Vanessa io	Southern Germany	*Urtica dioica*
Pieridae		
Pieris rapae	Berlin, Germany	*Brassica oleracea*
Saturniidae		
Actias selene	India	*Rhododendron* spp.
Attacus atlas	India	*Ligustrum vulgare*
Samia ricinii	India	*Ligustrum vulgare*
Automeris naranja	Southern America	*Quercus robur*
Lasiocampidae		
Odonestis pruni	Berlin, Germany	*Prunus domestica*
Gastropacha quercifolia	Berlin, Germany	*Prunus domestica*
Arctiidae		
Arctia caja	Southern Germany	*Taraxacum officinale*

Alkaloid injection

Alkaloids were either obtained commercially or had been isolated in our laboratory. Their purity was higher than 95% according GLC analysis (details in Theile 1998). Alkaloids were administered to larvae (usually first and second day of 5th instar) by injection using a microliter syringe (needle 0.11 mm inner diameter; 0.21 mm outer diameter). Alkaloids were dissolved in physiological salt solution adjusted to pH 6.5 to 7.0 and injected into the anterior abdominal pro-legs. Controls were treated with equal amounts of physiological salt solution without alkaloids. Injected larvae were allowed to feed on their established food plants or the artificial diet. Faeces were collected 6, 12, 48 and 78 h after treatment. Larvae were killed after 72 h by placing them into liquid nitrogen; they were stored at −20°C until further processing.

Alkaloid extraction

Alkaloid extraction (according Wink *et al.* 1995): Larvae or faeces were homogenised in 20 ml of 0.5 M hydrochloric acid and left standing for 2 h. The homogenate was adjusted to pH 12 with 6 M aqueous sodium-hydroxide solution. Alkaloids were extracted by solid phase extraction using Extrelut® columns (Merck Darmstadt) columns and dichloromethane as eluent. In order to convert alkaloid N-oxides to the corresponding free bases, zinc powder was added to the HCl extracts and they were incubated for 12 h prior to solid phase extraction. The amounts of N-oxides were determined indirectly by calculating the difference between reduced and non-reduced samples.

Capillary GLC and GLC-MS

The alkaloid extracts were separated and analysed by capillary GLC (Carlo Erbar 5100) or by a GLC-MS system consisting of a Carlo Erba 4160 GC which was coupled to a Finnigan 4500 quadrupole mass spectrometer. The separation conditions were:

Column type: DB 1; 15 m; 0.25 mm i.d.; 0.25 µm film thickness; split ratio 1:10; carrier gas: He; flow 1 ml/min; injector temperature: 250°C; oven temperature program: 120°C; 3 min isothermal; 100–300°C with a rate of 20°C/min; then 10 min isothermal. The electron impact mass spectra were recorded at 45 eV ionisation energy by an INCOS data-system. The Kovats index was determined by co-chromatography with a mixture of linear alkanes and by linear interpolation between the n-alkane signals in the gas-chromatogram. Alkaloids were identified according to their specific mass spectra and indicative RI values, determined earlier in our laboratory (Wink 1993c; Wink *et al.* 1995). Authentic reference alkaloids were used as external standards for quantification.

DNA isolation

Isolation and purification of total DNA from *Manduca sexta* and other sphingids was carried out following standard procedures (Sambrook *et al.* 1989; Wink & von Nickisch-Rosenegk 1997).

Amplification and sequencing of 16S rDNA

A partial section of the 16S rRNA gene with 528 nucleotides (nt) was amplified by Polymerase Chain Reaction (PCR) using the oligonucleotide primers: 5'CGC CTG TTT AA(T)C AAA AAC AT-3' and 5'-CTC CGG TT(C)T GAA CTC AGA TCA AGT-3'. PCR amplification was carried out in a volume of 50 µl Taq polymerase buffer containing 200 µM of each dNTP, 1 mM of each primer primer and approximately 1 µg total DNA. Addition of bovine serum albumin to the buffer at a final concentration of 10 to 20 µg/ml improved the efficiency of amplification. PCR was initiated by adding 1 unit of Taq polymerase (Amersham Pharmacia Biotech, Freiburg) to samples that had been heated at 94°C for 4 min. The cycling program consisted of 30 cycles with 45 s at 92°C, 45 s at 45°C and 1 min at 72°C performed on a Biometra thermocycler. Afterwards the reaction was treated with 1 U exonuclease and 2 U alkaline phosphatase to remove primers and non-incorporated nucleotides. 3 to 5 µl of 50 µl double-stranded PCR product were used to carry out a sequencing reaction according to the "Sequenase PCR direct sequencing kit" (USB-Amersham, Braunschweig). ^{35}S-dATP was chosen as a radioactive tracer. Sequencing primers were: 38: 5'AGA CGA GAA GAC CCT ATA GAG TTT TA-3'; 39b: 5'-TAA AAC TCT ATA GGG TCT TCT CGT CT-3' and 40: 5'-ATC ACC CCA ATA AAA TA-3'. Products of the sequencing reactions were separated on a 6% polyacrylamide/7 M urea gel by electrophoresis at 65 W. After drying, the gel was exposed to an x-ray film (Hyperfilm-MP, Amersham) for 3–4 days, and developed (X-ray developer and fixer, Kodak). About 350 nucleotides were readable per sequencing run. The sequence alignment was deduced from the reference sequence and secondary structure of *Drosophila yakuba* (Clary & Wolstenholme 1985).

Table 2 Toxicity of nicotine (approximate LD_{50} or LD_{100} values) in sphingids and other Lepidoptera

Art	Family	LD_{50} nicotine [mg/kg BW]	LD_{100} nicotine [mg/kg BW]
Laothoe populi	Sphingidae (SM)	–	50 (8)
Smerinthus ocellatus	Sphingidae (SM)	<200 (8)	–
Smerinthus o. atlanticus	Sphingidae (SM)	–	<500 (8)
Sphinx pinastri	Sphingidae (SP)	–	700 (11)
Sphinx ligustri	Sphingidae (SP)	400 (8)	700 (9)
Hyles euphorbiae	Sphingidae (M)	300–700 (15)	–
Deilephila elpenor	Sphingidae (M)	500–700 (9)	900 (9)
Acherontia atropos	Sphingidae (SP)	900 (6)	–
Hyles livornica	Sphingidae (M)	>900 (14)	–
Manduca sexta	Sphingidae (M)	1500 (12)	–
Vanessa io	Nymphalidae	–	50 (5)
Actias selene	Saturniidae	–	200 (11)
Odonestis pruni	Lasiocampidae	–	300 (7)
Pieris rapae	Pieridae	>200 (5)	–
Samia ricini	Saturniidae	–	500 (8)
Attacus atlas	Saturniidae	<300 (8)	–
Arctia caja	Arctiidae	<500 (5)	–
Automeris naranja	Saturniidae	–	700 (6)
Gastropacha quercifolia	Lasiocampidae	–	700 (6)

Alkaloids were administered by injection; toxicity was determined after 72 h. The respective number of larvae studied is given in parenthesis. M = Macroglossinae; SP = Sphinginae, SM = Smerinthinae. Taxa are ordered according to their degree of nicotine tolerance; – = not determined; BW = body weight

Table 3 Minimal recuperation times of larvae after nicotine injection

Species	Dose (mg/kg)	Minimal recuperation time (h)
Manduca sexta	500	0.5–2
Acherontia atropos	500	6–12
Hyles livornica	500	3.5–24
Deilephia elpenor	200	9–24
Hyles euphorbiae	500	32–72
Sphinx pinastri	500	6–24
Theratra alecto	200	24–48
Smerinthus ocellatus	200	5

Amplification and sequencing of nicotine binding sites of α-subunits of nAChR

The nicotine binding sites of α1- and α2-subunits of nAChR were amplified by PCR and sequenced directly as outline for 16S rDNA. PCR primer deduced from published sequences of *Drosophila* and *Schistocerca* (Hermans-Borgmeyer *et al.* 1986; Jonas *et al.* 1990; Sawruk *et al.* 1990; Marshall *et al.* 1990; Gundelfinger & Hess 1992), were: α1-subunits forward A1: 5′-GGC ATC(T) GAC CTG CG(A)T(C;G) GAG TAC A-3′ and 11: 5′-GAC(G) TAC TAC ATC TCG(A) GTG(C) g-3′; backward: A2: 5′ATC AGG TTG(A) ACC GTG TAG AAG(C) AG-3′; 12: 5′-CAG TGA T(C)GT T(G)GG G(C)GG AAT GAT T(C)TC-3′ and M6: 5′-ACC(G) AGC AA(T;C)C ATG GT AAC(G) AG-3′. α2-subunit: forward: M5: 5′-GAA GTT CGG C(A)TC C(G)TG GAC CTA CGA-3′; lll: 5′-GAC ATA TCG ACG AGG CGC GAG G-3′ and M9: 5′-TCG ACC TCT CCG AGT TTT ACA CC-3′; backward: A2 and M6.

Phylogenetic analyses

Three methods, MP (Maximum Parsimony), NJ (Neighbour Joining) and ML (Maximum Likelihood), were employed to analyse the sequence data. All analyses were performed with PAUP* 4.0b6a, (Swofford 2001). All heuristic searches for optimal trees were carried out by TBR (Tree-bisection-reconnection) branch swapping with option MULPARS in effect. In parsimony-based analyses, starting trees were obtained by stepwise addition. We checked that random addition of taxa did not lead to alternative, more parsimonious trees. Neighbour joining (distance-(minimum evolution) based) analyses were carried out using Jukes–Cantor distance or logdet algorithms. Analyses based on Maximum Likelihood used the following criteria: MP starting trees were obtained by heuristic search; number of substitution types = 2; transition/transversion ratio was estimated via ML; the distribution of rates at variable sites was assumed to follow a gamma distribution.

Results and discussion

Nicotine toxicity and nicotine tolerance

Nicotine was injected into the larvae of *Manduca sexta*, several other sphingids and other Lepidoptera to determine the degree of acute toxicity. Approximate LD_{50} or LD_{100} values are shown in Table 2. *Manduca sexta* was the most nicotine tolerant species tested, exhibiting an LD_{50} of 1.5 g nicotine/kg body weight (BW), followed by *Hyles livornica* and *Acherontia atropos* both with an LD_{50} of 900 mg/kg BW. Nicotine tolerance (defined here as LD_{100} above 500 mg nicotine/kg BW) (Table 2) is not restricted to *Manduca sexta* but is exhibited by several other sphingids of the subfamilies Macroglossinae and Sphinginae. Members of the Smerinthinae were more sensitive to nicotine and exhibited LD_{100} between 50 and < 500 mg nicotine/kg BW. Nicotine sensitivity (defined here as LD_{100} below 500 mg nicotine/kg BW) in the Smerenthinae was in the same range as that found for a few selected Lepidoptera of the families Nymphalidae, Saturniidae and Lasiocampidae. *Arctia caja*, an arctiid known to feed on alkaloid rich food plants, *Samia ricina*, *Automeris naranja* and *Gastropoda quercifolia* were the most tolerant species (outside the Sphingidae) towards nicotine with LD_{50} or LD_{100} values between 500 and 700 mg/kg BW; their tolerance was similar to that found in some members of the Sphinginae and Macroglossinae. It is remarkable that none of the nicotine-tolerant species, except *Manduca sexta*, naturally feed on nicotine containing host plants.

When doses higher than 500 mg/kg BW were injected, even larvae of *Manduca* and other tolerant species showed strong convulsions and became paralysed. The larvae either died within 30 h or recuperated and started feeding again. Typical minimal recuperation times (*i.e.* the duration after which larvae began crawling and feeding again) are listed in Table 3. This observation indicates that nicotine must have reached the nicotinic acetylcholine receptors in these species but that its content was reduced over time (by excretion and conjugation/degradation) until the animals were almost free from nicotine.

It should be recalled that nicotine is very toxic to vertebrates: in mice, LD_{50} was 0.3 mg/kg BW after intravenous injection, and 9.5 mg/kg BW after intraperitoneal injection. Oral toxicity is lower, with LD_{50} (in birds) of 17.8 mg/kg in *Agelaius* and 42 mg/kg BW

Species	Nicotine injected [mg/kg BW] (*N*)	Total recovery [%] (injected nicotine = 100%)	Recovery in faeces alkaloids [%] (recovered = 100%)	Recovery in larvae [%] (alkaloids recovered = 100%)
Manduca sexta	500 (5)	17.0 ± 5.9	100	0
Acherontia atropos	500/700/900 (6)	23.3 ± 3.6	83.2 ± 16.6	16.8 ± 16.6
Hyles livornica	500 (5)	15.9 ± 5.9	100	0
Deilephila elpenor	200 (5)	10.5 ± 6.1	100	0
Hyles euphorbiae	500 (3)	26.0 ± 11.5	85.4 ± 12.2	14.6 ± 12.2
Sphinx ligustri	300 (4)	14.1 ± 6.5	79.6 ± 20.4	20.4 ± 20.4
Sphinx pinastri	500 (3)	6.8 ± 3.1	100	0
Smerinthus ocellatus	200 (3)	7.4 ± 1.5	66.7 ± 33.3	33.3 ± 33.3

Table 4 Recovery of nicotine or metabolites from larvae after injection. *N* = number of individuals. Values are means ± standard error ($\bar{x} \pm$ s.e.)

Table 5 Metabolism of nicotine after injection

Species (alkaloid dose; *N*)	Total N-oxides [%] $\bar{x} \pm$ s.e.	Nicotine-N-oxide [%] $\bar{x} \pm$ s.e.	Cotinine-N-oxide [%] $\bar{x} \pm$ s.e.	Nicotine [%] $\bar{x} \pm$ s.e.	Cotinine [%] $\bar{x} \pm$ s.e.
Manduca sexta (500 mg/kg; $N = 5$)	47.2 ± 12.8	46.6 ± 13.0	0.6 ± 0.3	44.8 ± 14.8	8.0 ± 3.3
Acherontia atropos (500–700 mg/kg $N = 6$)	49.3 ± 12.6	45.2 ± 13.7	4.1 ± 1.8	49.8 ± 12.2	0.9 ± 0.5
Hyles livornica (500 mg/kg; $N = 5$)	37.7 ± 13.1	28.9 ± 11.7	8.8 ± 5.9	58.3 ± 11.9	4.0 ± 3.4
Deilephila elpenor (200 mg/kg; $N = 5$)	7.6 ± 3.8	6.3 ± 4.1	1.4 ± 1.4	58.2 ± 16.9	34.1 ± 14.0
Hyles euphorbiae (500 mg/kg; $N = 3$)	43.8 ± 9.4	22.8 ± 10.1	21.0 ± 18.4	50.8 ± 6.4	5.3 ± 4.1
Sphinx ligustri (300 mg/kg; $N = 5$)	21.5 ± 8.4	20.7 ± 8.3	0.8 ± 0.8	77.8 ± 8.6	0.7 ± 0.7
Sphinx pinastri (500 mg/kg; $N = 5$)	67.3 ± 32.7	67.3 ± 32.7	0	32.7 ± 32.7	0
Smerinthus ocellatus (200 mg/kg; $N = 3$)	37.0 ± 31.6	3.7 ± 3.7	33.3 ± 33.3	41.0 ± 25.9	21.9 ± 21.9

Total alkaloid content recovered was set 100%. Means $\pm$ standard error are given for each metabolite

in *Sturnus vulgaris* (reviewed in Wink 1993b); in humans nicotine is lethal at 1–2 mg/kg BW. Thus, *Manduca sexta*, most sphingids and several other Lepidoptera are 100 to 1000 times less sensitive to nicotine than humans (Table 2). It has been shown earlier that insect nervous tissue is protected by an ion-impermeable sheath which seems to be particularly efficient in *Manduca*. Since 98% of the nicotine and most other alkaloids applied would be present in an ionised form at pH 6.8 of *Manduca* hemolymph (Brattsten & Ahmad 1986), the charged molecules cannot reach the synapses by simple diffusion. Thus the ion-impermeable sheath could be responsible for relative nicotine insensitivity of all sphingids as observed in this study. Apparently the concentration of uncharged and diffusible alkaloid molecules is high enough at the elevated concentrations applied to reach the synapses and to modulate nAChR (see above).

Fate of nicotine in Lepidoptera larvae after injection

In order to find out whether the injected nicotine was sequestered by the larvae, excreted or degraded, faeces were collected within 72 h of nicotine injection. The total amount of nicotine and its metabolites recovered was in the range of 6.8 to 26% of the administered alkaloid dose (Table 4), indicating that 74 to 93% of nicotine was metabolised to polar conjugates or degradation products which are not detectable with the methods applied. The recoverable alkaloids were mostly found in the faeces and not in the larvae. That very small amounts of alkaloids (a few µg nicotine per larvae) were recovered from *Sphinx pinastri* and *Hyles euphorbiae* (Table 4) was probably due to the fact that these larvae had suffered strongly from nicotine injection; they were alive after 72 h but in bad physiological shape. These larvae could hardly move and had stopped feeding. Observations shown in Table 4 confirm earlier data that *Manduca* does not store nicotine as an acquired toxin (Self *et al.* 1964a,b; Morris 1983, 1984; Snyder *et al.* 1993, 1994; Murray *et al.* 1994) and indicate that Sphingidae are not among those insects that store large amounts of secondary metabolites found in their food plants for use as acquired defence compounds. However, the small transient amounts of nicotine in the hemolymph of *M. sexta* (or an undetected conjugation product) may protect from the parasite *Cotesia congregata* (Barbosa *et al.* 1991).

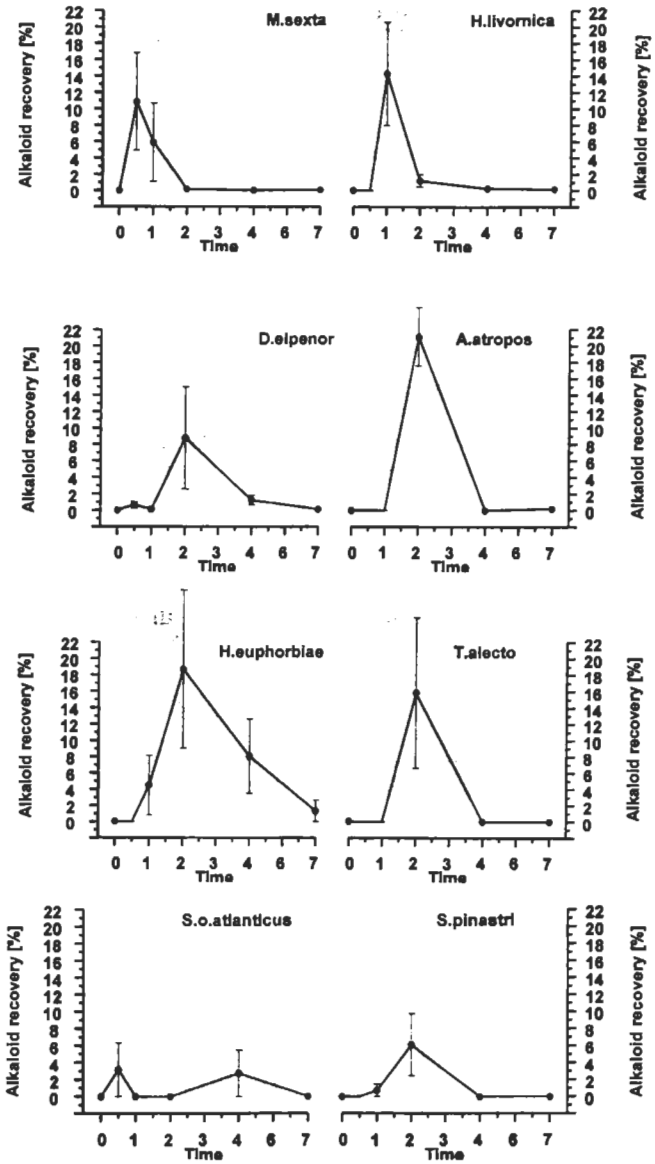


Fig. 1 Time course of alkaloid excretion in *Manduca sexta*. Nicotine was injected into the hemolymph. Alkaloids were extracted from the faeces at the time intervals (days) given. Alkaloid doses: *Manduca sexta*, *Hyles livornica* 500 mg/kg BW; *Deilephila elpenor* 200 mg/kg BW, *Acherontia atropos* 500 resp. 700 mg/kg BW, *Hyles euphorbiae* 500 mg/kg BW; *Theretra alecto*, *Smerinthus o. atlanticus* 200 mg/kg BW

Table 6 Inhibition of nicotine metabolism in *Manduca sexta* larvae by proadifen (SKF 525A)

	Nicotine	Nicotine/proadifen
Total recovery	16.8%	42.6%
Recovery in faeces	98.2%	99.9%
Recovery in larvae	1.8%	0.1%
Nicotine*	33.8%	98.6%
Nicotine N-oxide*	66.2%	1.4%
Cotinine*	0	0
Cotinine N-oxide*	0	0
Recuperation time	3.8 h	3.5 h

Larvae (from L3 stage onwards) were reared on an artificial diet containing 1% nicotine. 24 h prior to the experiments they were placed on nicotine-free diet, so that faeces were free of nicotine at the beginning of the experiments. Larvae obtained 600 mg nicotine/kg BW by injection (controls) as compared to 600 mg nicotine/kg BW plus 300 mg/kg BW proadifen. Proadifen alone did not exhibit toxic effects. Analysis of larvae and faeces was carried out after 48 h after injection
*Total recovery = 100%

In the faeces, N-oxides ranged between 7.6 and 67% of the recovered alkaloids (Table 5). The degree of nicotine oxidation into cotinine differed; it was low in nicotine tolerant species, such as *Manduca sexta*, and high in sensitive species, such as *Smerinthus ocellatus* (Table 5).

Kinetics of nicotine excretion

Since we administered the alkaloids by injection, any alkaloid that turned up in the faeces (Table 4) must have been eliminated via the Malpighian tubules by active excretion (Maddrell & Gardiner 1976). These results highlight the importance of nicotine excretion as a mechanism for alkaloid detoxification. Nicotine is a rather lipophilic alkaloid entering cells, including neuronal tissues, by free diffusion. It has been suggested that *mdr* proteins (which are ATP-dependent transporters known to pump out lipophilic compounds which have entered cells) play a key role in the excretion of nicotine (Murray *et al.* 1994).

In order to study the overall kinetics of alkaloid excretion, faeces were collected 6, 12, 48 and 72 h after alkaloid injections. Fig. 1 shows the results of a comparative experiment involving 8 species. Excretion of injected alkaloids started after 12 h and reached a maximum after 24 h; after 48 h alkaloids were almost completely eliminated. In *Manduca sexta* and *Hyles livornica*, which tolerated the highest amounts of nicotine (Tables 2 and 3), excretion sets in much earlier and reaches a maximum between 6 and 12 h after injection and is completed after 24 h.

Induction of nicotine tolerance

It has been reported that nicotine degrading enzymes, such as cytochrome oxidases (Brattsten & Ahmad 1986), are inducible by nicotine in *Manduca sexta* (Snyder *et al.* 1993, 1994). Since we carried out our studies with "naïve" caterpillars which had never been exposed to nicotine, we performed an additional experiment to assess the scope of the induction effect. Larvae of *Manduca sexta* were reared (from L3 stage onwards) on an artificial diet containing 1% nicotine. Both naïve and nicotine-reared larvae were injected with 1 g nicotine/kg BW. Alkaloid recovery rates (after 72 h) were 39.5 or 38.4%, respectively; *i.e.* they did not differ between the groups. Whereas naïve larvae excreted mostly nicotine (92% of total alkaloids), nicotine-induced larvae had 62% nicotine and 36% nicotine N-oxide as excretion products. In contrast to experiments described by Snyder *et al.* (1993, 1994), cotinine and cotinine N-oxide were hardly present in both naïve and induced larvae. A dramatic difference was observed however for the narcotic period after induction: it was 11 h for naïve animals and only 4.5 h for nicotine-induced larvae.

Table 7 Approximate LD_{50} and LD_{100} of different alkaloids administered by injection to *Manduca sexta* larvae

Alkaloid	LD_{50} of free base [mg/kg BW]	LD_{100} of the base [mg/kg BW]	Number of larvae studied
Nicotine	1500	–	12
Hyoscyamine	>1200	–	10
Pilocarpine	>1200	–	11
Sparteine	>1200	–	11
Brucine	>1000	–	7
Boldine	>1000	–	9
Senecionine	>1000	–	9
Ergometrine	1000	–	8
Emetine	500–1000	–	12
Cinchonidine	700	1000	9
Gramine	–	1000	9
Harmaline	–	700	15
Cytisine	500	700	11
Colchicine	–	500	11
Berberine	–	500	7
Sanguinarine	–	500	4
Arecoline	–	500	10
Aconitine	–	500	5
Physostigmine	<200	–	10

– = not determined

Table 8 Recovery and metabolisation of injected alkaloids in *Manduca sexta*. Alkaloids were injected in L5 larvae

Alkaloid	Alkaloid-dose [mg/kg BW]	Number of larvae	Total recovery [%] x ± s.e.	N-oxides [%] x ± s.e.	Metabolites [%] x ± s.e.	Recovery in faeces [%] x ± s.e.	Recovery in larvae [%] x ± s.e.
Nicotine	500	5	17.0 ± 5.9	47.2 ± 12.8	8.6	100 (3.1)	0
Hyoscyamine	1200	3	31.3 ± 8.9	25.7 ± 9.8	4.5	99.0 ± 0.7	1 ± 0.7
Sparteine	1200	4	38.3 ± 5.4	1.7 ± 1.5	–	68.6 ± 23.4	31.4 ± 23.4
Harmaline	500	3	58.8 ± 14.1	53.0 ± 9.6	–	64.7 ± 32.4	35.3 ± 32.4
Cinchonidine	700	3	70.0 ± 7.9	22.0 ± 4.3	–	62.6 ± 20.8	37.4 ± 20.8
Cytisine	500	4	20.2 ± 10.1	17.6 ± 11.2	–	100	0
Senecionine	1000	4	39.3 ± 3.6	12.4 ± 4.6	–	96.8 ± 1.3	3.2 ± 1.3

Data are given as mean ± standard error

Application of 300 mg/kg BW of the cytochrome inhibitor SKF 525A (proadifen) (Gorrod & Jenner 1975; Snyder *et al.* 1993) reduces the formation of nicotine N-oxide and the rate of alkaloid degradation in *Manduca sexta* (Table 6): Instead of a total alkaloid recovery of 16.8%, as in nicotine-treated larvae, total alkaloids increased to 42.6% in SKF 525A/nicotine treated larvae. Whereas nicotine N-oxide levels were high in normal larvae they were substantially reduced in SKF 525A treated larvae, clearly indicating that SKF 525A acts as an inhibitor of cytochrome oxidases. However, nicotine tolerance was apparently identical in both groups (Table 6).

The kinetics of nicotine excretion were enhanced in induced larvae compared to naïve larvae: about 6–12 h after nicotine injection (1 g/kg BW), alkaloids were completely excreted in induced larvae, whereas naïve larvae excreted alkaloids for 24 h; *i.e.* nicotine induc-

Table 9 Coding nucleotide sequences of MSα1 and MSα2 from nAChR of *Manduca sexta*

	Sequence	Position
MSα1		486
MSα2	G G G T T T C A G ↓ G T A G A T C T G C G A C A T A T C G A T	
MSα1		516
MSα2	G A A G T G C G A G G C A C C A A C G T C G T G G A G T T G	
MSα1	C A T C T C G	546
MSα2	G G C G T C G A C C T C T C C G A G T T T T A C A C C T C T	
MSα1	G T G G A A T G G G A C A T A A T G C G A G T G C C G G C G	576
MSα2	G T C G A G T G G G A C A T C C T G G A G G T G C C C G C C	
MSα1	A C G A G ↓ A A A C G A G A A A T T C T A C T C C T G C T G C	606
MSα2	G T C C G A A A T G A A A A G T T C T A C A C G T G C T G C	
MSα1	G A G G A A C C A T A C C C C G A C A T C A T C T T C A A C	636
MSα2	G A C G A G C C C T A T C T A G A T A T A A C T T T C A A C	
MSα1	A T A A C G C T C A G G A G A A A A A C C C T A T T C T A C	696
MSα2	A T C A C G A T G C G C C G C A A A A C A C T C T T C T A C	
MSα1	A C G G T C A A T C T G A T C A T C C C T T G C G T C G G A	726
MSα2	A C C G T T A A T C T G A T C A T T C C G T G C A T G G G A	
MSα1	A T A T C T T T C C T A T C A G T T T T A G T C T T C T A C	756
MSα2	A T A T C G T T C C T G A C G G T C C T G G T A T T T T A C	
MSα1	T T A C C T T C G G A T T C G G G G G A G A A A A T C T C G	786
MSα2	T T G C C T T C G G A C A G T G G C G A G A A G G T A T C G	
MSα1	C T C T G T A T A T C C A T T C T T C T G T C A T T G A C C	816
MSα2	C T G T C C A T A T C G A T C C T A C T G T C G C T C A C C	
MSα1	G T G T T C T T T T T G T T G T T A G C A G A A A T C A T T	846
MSα2	G T G T T C T T C C T G C T G T T G G C C G A G A T C A T T	
MSα1	C C G C C A A C A T C A C T G A C T G T C C C G T T A T T G	876
MSα2	C C A C C G A C T T C G C T G G T G G T G C C A C T G C T T	
MSα1	G G A A A A T A T T T G	888
MSα2	G G C A A G T T T G T T	

Positions of both introns in MSα2 are indicated with ↓

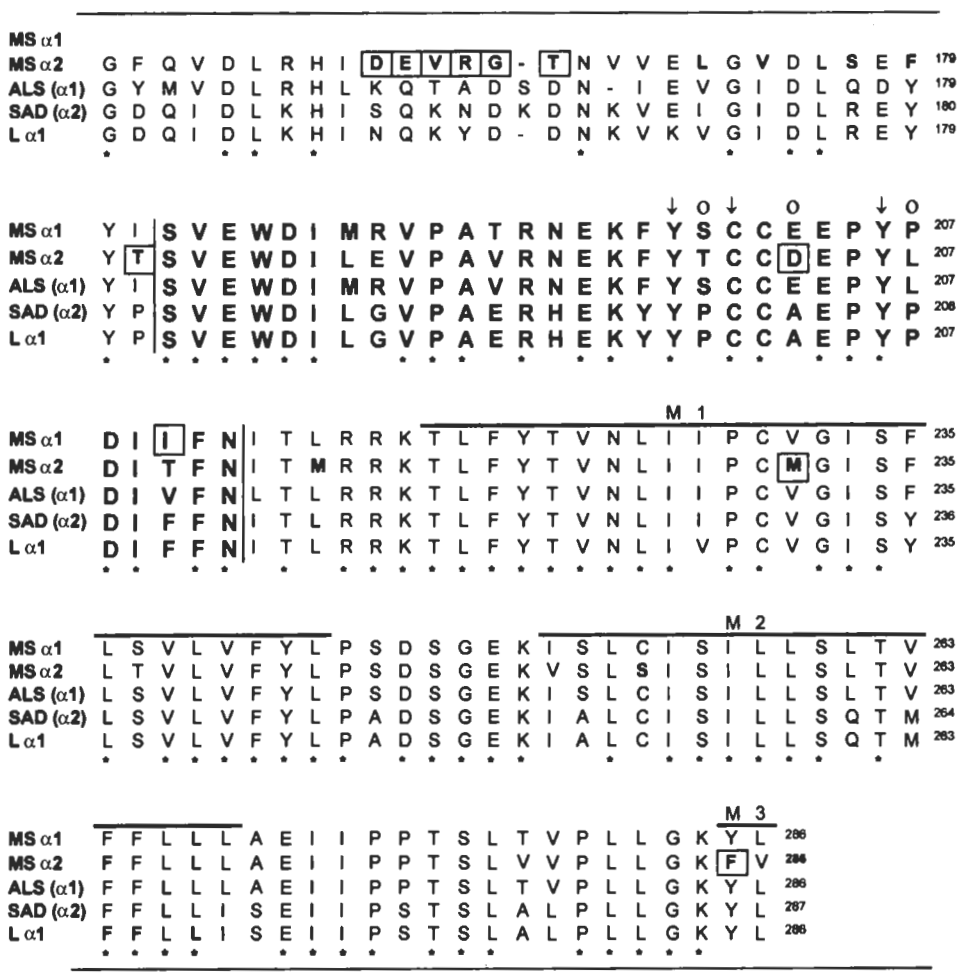
tion apparently also stimulated the rate of alkaloid excretion. The cytochrome inhibitor SKF 525A had no influence on excretion kinetics. From other animals and humans it is known that ABC transporters and *mdr* proteins are inducible and important for the elimination of xenobiotics (Fromm 2000; LeBlanc 1994; Krishna and Mayer 2000; Smital & Kurelec 1998). Thus, both alkaloid metabolism and alkaloid excretion appear to be inducible traits in *Manduca sexta* and probably in the other nicotine tolerant Sphingids.

Manduca sexta *can also tolerate other alkaloids which affect different molecular targets*

Manduca sexta not only feeds on tobacco plants in nature, but also on many other members of the Solanaceae, such as *Physalis*, *Datura*, and *Solanum* (Hodges 1971; Holloway *et al.* 1987; Watson & Whalley 1983). These host plants also contain active alkaloids, albeit of different classes and activities than Nicotiana. Whereas hyoscyamine, a major alkaloid from *Datura*, inhibits muscarinic acetylcholine receptors (mAChR), the steroidal alkaloid solanine (present in *Solanum*) is active at biomembranes and neuroreceptors (Wink *et al.* 1998). As these alkaloids affect other targets as compared to nicotine, we were interested to study the reaction of *Manduca sexta* larvae towards injection of different alkaloids known to be toxic in most animals (Wink 1993b) but affecting different molecular targets (Wink 2000). Most of these alkaloids exhibit LD_{50} values of 1 to 100 mg/kg BW after i.p. or i.v. application in vertebrates (Wink 1993b).

Experiments to determine approximate LD_{50} or LD_{100} values (Table 7) were performed in analogy to the nicotine assays, shown in Table 2. Except for physostigmine, a potent acetylcholine esterase inhibitor, *Manduca sexta* is able to tolerate the other alkaloids to a remarkable degree and LD_{50}/LD_{100} values were usu-

Table 10 Comparisons of amino acid sequences of α-subunits in insects



MSα1, MSα2 = α1, α2 subunits of *Manduca sexta*; ALS = α1-subunit of *Drosophila*; SAD = α2 subunit of *Drosophila*; Lα1 = α1-subunit of *Schistocerca gregaria*. The nicotine binding site is bordered by vertical lines. Transmembrane regions are marked with a horizontal line. ↓ = indicates amino acids which are thought to be important for nicotine binding. ○ = indicates amino acids which are apparently important for nicotine tolerance

M.sexta	T	C	G	G	T	G	G	A	A	T	G	G	G	A	C	A	T	A	A	T	G	C	G	A	G	T	G	C	C
S.pinastri	?	?	?	?	?	?	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S.ligustri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.atropos	.	.	.	?	?	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.
H.livornica	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H.hippophaes	?	?	?	?	?	?	?	.	.	.	.	.	.	.	.	.	?	?	?	.	.	.	.	C	.	.	.	.	.
H.euphorbiae	?	?	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	?	.	.	.	C	.	.	.	.	.
H.e.conspicua	?	?	?	?	?	?	?	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
D.nerii	.	.	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
L.populi	?	?	?	?	?	?	?	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.
L.austauti	?	?	?	?	?	?	?	?	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.
S.ocellatus	?	?	?	?	?	?	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
S.o.atlanticus	.	.	.	.	?	?	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
D.elpenor	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H.fuciformis	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.

M.sexta	G	G	C	G	A	C	G	A	G	A	A	A	C	G	A	G	A	A	A	T	T	C	T	A	C	T	C	C	T
S.pinastri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S.ligustri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.atropos	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H.livornica	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.
H.hippophaes	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H.euphorbiae	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H.e.conspicua	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
D.nerii	A	.	.	A	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L.populi	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.
L.austauti	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.
S.ocellatus	.	.	.	T	.	.	A	.	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.
S.o.atlanticus	.	.	.	T	.	.	A	.	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.
D.elpenor	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H.fuciformis	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

M.sexta	G	C	T	G	C	G	A	G	G	A	A	C	C	A	T	A	C	C	C	C	G	A	C	A	T	C	A	T	C
S.pinastri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
S.ligustri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.
A.atropos	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.
H.livornica	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H.hippophaes	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H.euphorbiae	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H.e.conspicua	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D.nerii	.	T	.	.	.	.	.	A	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
L.populi	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
L.austauti	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
S.ocellatus	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.
S.o.atlanticus	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.
D.elpenor	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A
H.fuciformis	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A

M.sexta	T	T	C	A	A	C
S.pinastri	.	.	.	.	.	.
S.ligustri	.	.	.	.	.	.
A.atropos	.	.	.	.	.	.
H.livornica	.	.	.	.	.	.
H.hippophaes	.	.	.	.	.	.
H.euphorbiae	.	.	.	.	.	.
H.e.conspicua	.	.	.	.	.	.
D.nerii	.	.	T	.	.	.
L.populi	.	.	.	.	.	.
L.austauti	.	.	.	.	.	.
S.ocellatus	.	.	.	.	.	.
S.o.atlanticus	.	.	.	.	.	.
D.elpenor	.	.	.	.	.	.
H.fuciformis	.	.	.	.	.	.

Table 11 Nucleotide sequence of the acetylcholine binding site of the α1-subunit (positions 182–212) of nAChR sphingids

Nucleotides identical to those of *Manduca sexta* are marked as ·

ally above 500 and 700 mg/kg BW; *i.e.* much higher than in vertebrates.

For a number of alkaloids tested in Table 7 we were able to determine alkaloid recovery rates and metabolisation using GLC and GLC-MS (Table 8). For most alkaloids between 80 and 40% of the initial dose could not be recovered, indicating degradation and excretion as important mechanisms. Alkaloids were mainly recovered from the faeces; the recovery of sparteine, harmaline and cinchonidine in larvae was probably due to toxic effects, as larvae were very weak after injection and stopped feeding altogether. Metabolisation of the injected alkaloids into N-oxides was observed for hyoscyamine, harmaline, cinchonidine, cytisine and senecionine (Table 8). We assume that the mechanisms that help *Manduca* tolerate most of these otherwise toxic alkaloids are similar to those discussed for nicotine above. These mechanisms would thus not be nicotine-specific but cover a wide range of alkaloids.

Molecular characterisation of the acetylcholine binding sites of nicotinic acetylcholine receptors in Manduca sexta

Previous experiments have shown a relative (up to 100-fold) insensitivity of *Manduca sexta* neurons towards nicotine as compared to nicotine-sensitive neurons of *Periplaneta americana* (Sattelle 1980; Morris 1984; David & Sattelle 1984). Since the observed tolerance of *Manduca sexta* and other sphingids towards nicotine (Tables 2–7) could be due to a modification of the acetylcholine/nicotine binding sites of $\alpha 1$ or $\alpha 2$ subunits of nAChR, we tried to determine and to compare the corresponding amino acid sequences of *Manduca* and other nicotine tolerant and nicotine sensitive insects.

Known DNA sequences of *Drosophila melanogaster* and *Schistocerca gregaria* were selected to develop PCR

	T	C	T	G	T	C	G	A	G	T	G	G	G	A	C	A	T	C	C	T	G	G	A	G	G	T	G	C	C
M.sexta																													
S.pinastri	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S.ligustri	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.atropos	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.	.	.	.	.
H.livornica	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.
H.hippophaes	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.
H.euphorbiae	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.
H.e.conspicua	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.
D.nerii	.	.	C	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
M.tiliae	?	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
L.populi	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.
L.austauti	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.
S.ocellatus	.	.	C	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	A	.	.
D.elpenor	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
H.fuciformis	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	C	G	C	C	G	T	C	C	G	A	A	A	T	G	A	A	A	A	G	T	T	C	T	A	C	A	C	G	T
M.sexta																													
S.pinastri	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S.ligustri	.	.	.	?	.	.	.	.	?	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.atropos	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H.livornica	.	.	.	.	.	.	G	.	.	G	.	.	C	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.
H.hippophaes	.	.	.	.	.	.	G	.	.	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
H.euphorbiae	.	.	.	.	.	.	G	.	.	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
H.e.conspicua	.	.	.	.	.	.	G	.	.	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
D.nerii	.	.	.	?	.	.	G	.	?	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
M.tiliae	T	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L.populi	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.
L.austauti	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.
S.ocellatus	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D.elpenor	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H.fuciformis	T	.	.	.	.	.	A	.	.	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.

	G	C	T	G	C	G	A	C	G	A	G	C	C	C	T	A	T	C	T	A	G	A	T	A	T	A	A	C	T
M.sexta																													
S.pinastri	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	G	.	.	.	.	.	.	.	.	A
S.ligustri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	A
A.atropos	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	C	.	.	.	.	.	G
H.livornica	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	T	.	.	.	G
H.hippophaes	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	G
H.euphorbiae	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	T	.	.	.	G
H.e.conspicua	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	T	.	.	.	G
D.nerii	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	C	.	.	C	.	.	.	.	.	A
M.tiliae	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	G
L.populi	.	T	.	T	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	C	.	.	.	.	.	A
L.austauti	.	T	.	T	.	.	T	.	.	.	.	.	.	?	.	.	C	.	.	G	.	.	C	.	.	.	?	.	A
S.ocellatus	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	T	.	.	C	.	.	.	.	.	A
D.elpenor	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	G
H.fuciformis	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	A

	T	T	C	A	A	C
M.sexta						
S.pinastri	.	.	.	.	.	.
S.ligustri	.	.	.	.	.	.
A.atropos	.	.	.	.	.	.
H.livornica	.	.	T	.	.	.
H.hippophaes	.	.	T	.	.	.
H.euphorbiae	.	.	T	.	.	.
H.e.conspicua	.	.	?	.	.	.
D.nerii	.	.	.	.	.	.
M.tiliae	.	.	.	.	.	.
L.populi	.	.	T	.	.	.
L.austauti	.	.	?	?	?	?
S.ocellatus	.	.	T	.	.	.
D.elpenor	.	.	.	.	.	.
H.fuciformis	.	.	T	.	.	.

Table 12 Nucleotide sequence of the acetylcholine binding site of the α-2 subunit (positions 182–212) of nAChR sphingids

Nucleotides identical to those of *Manduca sexta* are marked as ·

Molecular characterisation of the acetylcholine binding sites of nicotinic acetylcholine receptors in Manduca sexta

Previous experiments have shown a relative (up to 100-fold) insensitivity of *Manduca sexta* neurons towards nicotine as compared to nicotine-sensitive neurons of *Periplaneta americana* (Sattelle 1980; Morris 1984; David & Sattelle 1984). Since the observed tolerance of *Manduca sexta* and other sphingids towards nicotine (Tables 2–7) could be due to a modification of the acetylcholine/nicotine binding sites of $\alpha 1$ or $\alpha 2$ subunits of nAChR, we tried to determine and to compare the corresponding amino acid sequences of *Manduca* and other nicotine tolerant and nicotine sensitive insects.

Known DNA sequences of *Drosophila melanogaster* and *Schistocerca gregaria* were selected to develop PCR

M.sexta	T	C	T	G	T	C	G	A	G	T	G	G	G	A	C	A	T	C	C	T	G	G	A	G	G	T	G	C	C
S.pinastri	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S.ligustri	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.atropos	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.	.	.	.	.
H.livornica	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.
H.hippophaes	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.
H.euphorbiae	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.
H.e.conspicua	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.
D.nerii	.	.	C	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
M.tiliae	?	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
L.populi	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.
L.austauti	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.
S.ocellatus	.	.	C	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	A	.
D.elpenor	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
H.fuciformis	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

M.sexta	C	G	C	C	G	T	C	C	G	A	A	A	T	G	A	A	A	A	G	T	T	C	T	A	C	A	C	G	T
S.pinastri	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S.ligustri	.	.	.	?	.	.	.	.	?	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.atropos	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H.livornica	.	.	.	.	.	.	G	.	.	G	.	.	C	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.
H.hippophaes	.	.	.	.	.	.	G	.	.	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
H.euphorbiae	.	.	.	.	.	.	G	.	.	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
H.e.conspicua	.	.	.	.	.	.	G	.	.	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
D.nerii	.	.	.	?	.	.	G	.	?	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
M.tiliae	T	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L.populi	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.
L.austauti	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.
S.ocellatus	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D.elpenor	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H.fuciformis	T	.	.	.	.	.	A	.	.	G	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.

M.sexta	G	C	T	G	C	G	A	C	G	A	G	C	C	C	T	A	T	C	T	A	G	A	T	A	T	A	A	C	T
S.pinastri	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	G	.	.	.	.	.	.	.	.	A
S.ligustri	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	A
A.atropos	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	G	.	.	C	.	.	.	.	.	G
H.livornica	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	T	.	.	G
H.hippophaes	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	C	.	.	G
H.euphorbiae	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	T	.	.	G
H.e.conspicua	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	T	.	.	G
D.nerii	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	C	.	.	C	.	.	.	.	.	A
M.tiliae	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	G
L.populi	.	T	.	T	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	C	.	.	.	.	.	A
L.austauti	.	T	.	T	.	.	T	.	.	.	.	.	.	?	.	.	C	.	.	G	.	.	C	.	.	.	?	.	A
S.ocellatus	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	T	.	.	C	.	.	.	.	.	A
D.elpenor	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	G
H.fuciformis	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	C	.	.	T	.	.	.	.	.	T	.	.	A

M.sexta	T	T	C	A	A	C
S.pinastri	.	.	.	.	.	.
S.ligustri	.	.	.	.	.	.
A.atropos	.	.	.	.	.	.
H.livornica	.	.	T	.	.	.
H.hippophaes	.	.	T	.	.	.
H.euphorbiae	.	.	T	.	.	.
H.e.conspicua	.	.	?	.	.	.
D.nerii	.	.	.	.	.	.
M.tiliae	.	.	.	.	.	.
L.populi	.	.	T	.	.	.
L.austauti	.	.	?	?	?	?
S.ocellatus	.	.	T	.	.	.
D.elpenor	.	.	.	.	.	.
H.fuciformis	.	.	T	.	.	.

Table 12 Nucleotide sequence of the acetylcholine binding site of the α-2 subunit (positions 182–212) of nAChR sphingids

Nucleotides identical to those of *Manduca sexta* are marked as ·

Drosophila ALS	S	V	E	W	D	I	M	R	V	P	A	V	R	N	E	K
Drosophila SAD	.	.	.	.	.	.	L	G	.	.	.	E	.	H	.	.
S. gregaria αL1	.	.	.	.	.	.	L	G	.	.	.	E	.	H	.	.
M. sexta	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
S. pinastri	?	?	?	.	.	.	.	.	.	.	.	T	.	.	.	.
S. ligustri	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
A. atropos	.	?	.	.	.	.	.	.	?	.	.	T	.	.	.	.
H. livornica	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
H. hippophaes	?	?	?	.	.	?	.	.	.	.	.	T	.	.	.	.
H. euphorbiae	?	.	.	.	.	.	?	.	.	.	.	T	.	.	.	.
H. e. conspicua	?	?	?	.	.	.	.	.	.	.	.	T	.	.	.	.
D. nerii	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
L. populi	?	?	?	.	.	.	.	.	.	.	.	T	.	.	.	.
L. austauti	?	?	?	.	.	.	.	.	.	.	.	T	.	.	.	.
S. ocellatus	?	?	?	.	.	.	.	.	.	.	.	T	.	.	.	.
S. o. atlanticus	.	?	?	.	.	.	.	.	.	.	.	T	.	.	.	.
D. elpenor	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
H. fuciformis	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.

Drosophila ALS	F	Y	S	C	C	E	E	P	Y	L	D	I	V	F	N
Drosophila SAD	Y	.	P	.	.	A	.	.	.	P	.	.	F	.	.
S. gregaria αL1	Y	.	P	.	.	A	.	.	.	P	.	.	F	.	.
M. sexta	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
S. pinastri	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
S. ligustri	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
A. atropos	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
H. livornica	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
H. hippophaes	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
H. euphorbiae	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
H. e. conspicua	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
D. nerii	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
L. populi	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
L. austauti	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
S. ocellatus	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
S. o. atlanticus	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
D. elpenor	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
H. fuciformis	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.

Table 13 Amino acid sequence of the acetylcholine binding site of the α-1 subunit (positions 182–212) of nAChR of sphingids in comparison to those of *Drosophila melanogaster* and *Schistocerca gregaria*

Identical amino acids are marked as ·

primers in order to amplify the acetylcholine binding domain of α1 and α2 subunits of nAChR from *Manduca sexta*, which we termed Msα1 and Msα2 (Table 9). The coding region of Msα1 contained 107 and Msα2 and 134 amino acids (corresponding to position 150 to 286 of the *Drosophila* or *Schistocerca* subunit; Table 10). The amplified region covers a large part of the N-terminus and the transmembrane regions M1 and M2 (Table 10). In the amplified Msα2 product two introns of 76 and 87 nucleotide length were found (Theile 1998), which were also seen in Dα2 subunit of *Drosophila*. As these introns are of no importance for our study, we did not document their sequences in the subsequent sequence alignments.

Several studies on α subunits of AChR from *Torpedo* and insects have clearly shown that acetylcholine/nicotine binding sites are positioned between amino acid residues 173 and 204 (Kao *et al.* 1984; Lentz 1995; Middleton & Cohen 1991; Mishina *et al.* 1985). The relevant amino acid positions in *Manduca* would be positions between Ser 182 and Asn 212 (Table 10). According to Luetje *et al.* (1993) positions 200, 203 and 207 are important for nicotine sensitivity and positions Tyr 199, Cys 201 and Tyr 206 for nicotine binding. Whereas these important amino acids are identical in *Manduca* and in nicotine sensitive *Drosophila* and *Schistorcerca*, a few differences were seen at other positions. A number of amino acid substitutions can be seen in Msα2 that are not present in *Drosophila*, *Schistocerca* or Msα1 from *Manduca*; however, we consider it unlikely that these mutations confer nicotine tolerance (Table 10).

We also amplified the binding sites of both subunits from 14 other sphingids whose sensitivity towards nicotine was determined in this study. Tables 11–14 document the nucleotide sequences and the amino acid sequences of the putative nicotine binding sites. The amino acid composition of α1 and α2 subunits is identical in all sphingids studied with the corresponding *Manduca* subunits. Because no apparent amino acid substiution can be seen in the nicotine binding site of the acetylcholine receptor of nicotine tolerant and

nicotine sensitive sphingids, a simple target site modification as found in the Monarch (Holzinger & Wink 1996) can be ruled out. Thus, it is likely that detoxification and excretion and not target-site mutations mediate alkaloid tolerance in Sphingidae.

In conclusion, *Manduca sexta* and many other Sphingidae of the Macroglossinae and Sphinginae tolerate high doses of nicotine and other alkaloids, which are usually toxic to animals. An inducible degradation mechanism, coupled with a rapid and inducible excretion system, appear to help Sphingidae to tolerate a wide range of toxins and thus to live on host plants rich in otherwise toxic secondary metabolites.

Reconstruction of molecular phylogenies of Sphingids

In the last part of our study we investigated how nicotine tolerance might have evolved. As a mitochondrial marker gene to provide the framework to test these evolutionary questions we sequenced approx. 450 nucleotides of 16S rRNA gene from 17 sphingids (Table 15). Adenine (A) and thymine (T) were the most abundant nucleotide with a frequency of 39.1 and 38.8%, respectively, followed by guanine (14.2%) and cytosine (7.9%). The predominance of AT is typical for mitochondrial genes of insects (Schneider *et al.* 1999). Of 442 characters 100 were parsimony informative, whereas 312 positions were invariable and 30 variable but uninformative. Trees reconstructed with MP, NJ and ML showed similar topologies (Fig. 2). Minet (1994) and Kitching & Cadiou (2000) had recognised three subfamilies Macroglossinae, Sphinginae and Smerinthinae, whereas Hodges (1971) had treated the two latter subfamilies as tribes within the Sphinginae. Although the sequence data do not resolve the basal relationships, they would be in accordance with the view of Minet (1994) to recognize the three subfamilies as monophyletic groups. We tentatively assume that the Smerenthinae cluster as a sister group to the Sphinginae and that Macroglossinae form a monophyletic unit

Table 14 Amino acid sequence of the acetylcholine binding site of the α-2 subunit (positions 182–212) of nAChR of sphingids in comparison to those of *Drosophila melanogaster* and *Schistocerca gregaria*

Drosophila ALS	S	V	E	W	D	I	M	R	V	P	A	V	R	N	E	K
Drosophila SAD	.	.	.	.	.	.	L	G	.	.	.	E	.	H	.	.
S.gregaria αL1	.	.	.	.	.	.	L	G	.	.	.	E	.	H	.	.
M.sexta	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
S.pinastri	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
S.ligustri	.	.	.	.	.	.	L	E	.	.	.	.	?	.	.	.
A.atropos	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
H.livornica	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
H.hippophaes	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
H.euphorbiae	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
H.e.conspicua	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
D.nerii	.	.	.	.	.	.	L	E	.	.	.	.	?	.	.	.
M.tiliae	?	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
L.populi	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
L.austauti	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
S.ocellatus	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
D.elpenor	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.
H fuciformis	.	.	.	.	.	.	L	E	.	.	.	.	.	.	.	.

Drosophila ALS	F	Y	S	C	C	E	E	P	Y	L	D	I	V	F	N
Drosophila SAD	Y	.	P	.	.	A	.	.	.	P	.	.	F	.	.
S.gregaria αL1	Y	.	P	.	.	A	.	.	.	P	.	.	F	.	.
M.sexta	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
S.pinastri	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
S.ligustri	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
A.atropos	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
H.livornica	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
H.hippophaes	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
H.euphorbiae	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
H.e.conspicua	.	.	T	.	.	D	.	.	.	.	.	.	T	?	.
D.nerii	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
M.tiliae	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
L.populi	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
L.austauti	.	.	T	.	.	D	.	.	.	.	.	.	T	?	?
S.ocellatus	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
D.elpenor	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.
H.fuciformis	.	.	T	.	.	D	.	.	.	.	.	.	T	.	.

Identical amino acids are marked as ·

Table 15 Partial nucleotide sequences of 16S rDNA of Sphingidae

```
Manduca_sexta             TCTAATCTGCCCACTGAT??AATAATTGAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Hyles_livornica           TCTAATCTGCCCACTGATTTAATAATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Hyles_euphorbiae          TCTAATCTGCCCACTGATTTAATAATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Hyles_e_conspicua         TCTAATCTGCCCACTGATTTAATAATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Hyles_hippophaes          TCTAATCTGCCCACTGATTTAATAATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Deilephila_elpenor        TCTAATCTGCCCACTGATTTAAAAATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Hemaris_fuciformis        TCTAATCTGCCCACTGATTTAATTATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Daphnis_nerii             TCTAATCTGCCCACTGATT??TTTAATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Proserpinus_proserpina    TCTAATCTGCCCACTGATT??TTAATTGAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Acherontia_atropos        TCTAATCTGCCCACTGATTTAATAATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Sphinx_pinastri           TCTAATCTGCCCACTGAT??AAAAATTGAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Sphinx_ligustri           TCTAATCTGCCCACTGAT??ATAAATTGAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Mimas_tiliae              TCTAATCTGCCCACTGATG?AAACATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTA
Smerinthus_ocellata       TCTAATCTGCCCACTGATTTAAAAATTAAAGGGCTGCAGTATTTTGACTGTACAAAGGTA
Smerinthus_o_atlantica    TCTAATCTGCCCACTGATTTAAAAATTAAAGGGCTGCAGTATTTTGACTGTACAAAGGTA
Laothoe_populi            TCTAATCTGCCCACTGATTAAATAATTAAAGGGCTGCAGTATTTTGACTGTACAAAGGTA
Laothoe_austauti          TCTAATCTGCCCACTGATTATATAATTAAAGGGCTGCAGTATTTTGACTGTACAAAGGTA
Graellsia_isabellae       TCTAATCTGCCCACTGAT?AATTTATTAAAGGGCTGCAGTATTTTGACTGTACAAAGGTA

Manduca_sexta             GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAAATTG
Hyles_livornica           GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAGATTG
Hyles_euphorbiae          GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAGATTG
Hyles_e_conspicua         GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAGATTG
Hyles_hippophaes          GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAGATTG
Deilephila_elpenor        GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAGATATAAGTTG
Hemaris_fuciformis        GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAGATATAAATTG
Daphnis_nerii             GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAAATTG
Proserpinus_proserpina    GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAAANTG
Acherontia_atropos        GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAGATTG
Sphinx_pinastri           GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAGGATTGGATGAAATATAAATTG
Sphinx_ligustri           GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAGGATTGGATGAAATATAAATTG
Mimas_tiliae              GCATAATCAATAGTCTCTTAATTGGTGACTTGTATGAAGGATTGGATGAAATATAAATTG
Smerinthus_ocellata       GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAAATTG
Smerinthus_o_atlantica    GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAAATTG
Laothoe_populi            GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAAATTG
Laothoe_austauti          GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAAATTG
Graellsia_isabellae       GCATAATCATTAGTCTCTTAATTGGTGACTTGTATGAAGGATTGGATGAGATATAAACTG

Manduca_sexta             TCTCTATTATATATA--ATTGAATTTAATTTTTTAATTAAAAAGTTAAAATATATTAAAA
Hyles_livornica           TCTCTAAAATATTAA---TAGAAATTAATTTTTTAATTAAAAAGTTAAAATAAATTAAAA
Hyles_euphorbiae          TCTCTAAAATATTAA---TAGAAATTAATTTTTTAATTAAAAAGTTAAAATAAATTAAAA
Hyles_e_conspicua         TCTCTAAAATATTAA---TAGAAATTAATTTTTTAATTAAAAAGTTAAAATAAATTAAAA
Hyles_hippophaes          TCTCTAAAATATTAG---TAGAAATTAATTTTTTAATTAAAAAGTTAAAATAAATTAAAA
Deilephila_elpenor        TCTCTAAAATATTTA---TAGAAATTAATTTTTTAATTAAAAAGTTAAAATAAATTAAAA
Hemaris_fuciformis        TCTCTTAAATATTAG---TTGAATTTAATTTTTTAATTAAAAAGTTAAAATTATTTAAAA
Daphnis_nerii             TCTCTAAGATATTTA---TTGAATTTAATTTTTTAATTAAAAAGTTAAAATAAGTTAAAA
Proserpinus_proserpina    TCTCTATAATATAAA---TTGAATTTAATTTTTTAATTAAAAAGTTAAAATGAATTAAAA
Acherontia_atropos        TCTCTATAATATTAA--TTTGAATTTAATTTTTAAATTAAAAAGTTTAAATATTTTAAAA
Sphinx_pinastri           TCTCTAATATATATA--ATTGAATTTAATTTTTTAATTAAAAAGTTAAAATATATTAAAA
Sphinx_ligustri           TCTCTAATGTATATA--GTTGAATTTAATTTTTTAATTAAAAAGTTAAAATGTATTAAAA
Mimas_tiliae              TCTCTAAAGTATATA--ATTGAAATTAATTTTTTAATTAAAAAGTTAAAATAATTTAAAA
Smerinthus_ocellata       TCTCTAAAATATTAA--TTTGAATTTAATTTTTTGGTTAAAAAGCTAAAATATTTTAAAA
Smerinthus_o_atlantica    TCTCTAATATATTAA--TTTGAATTTAATTTTTTGGTTAAAAAGCTAAAATATTTTAAAA
Laothoe_populi            TCTCTAAGATATTGA--ATTGAATTTAATTTTTTAATTAAAAAGTTAAAATATTTTAAAA
Laothoe_austauti          TCTCTAAAATATTAA--ATTGAATTTAATTTTTTAATTAAAAAGTTAAAATATTTTAAAA
Graellsia_isabellae       TCTTAAAGGTAAATAAAATTGAATTTAATTTTTTAATCAAAAAGTTAAAATATATTAAAA

Manduca_sexta             AGACGAGAAGACCCTATAGAGTTTTATATTTTTTATTTTTAAAATTATAAATTTATATTA
Hyles_livornica           AGACGAGAAGACCCTATAGAGTTTTATATTTATTTATTTTTAAATTTTTAATTAA?TTGA
Hyles_euphorbiae          AGACGAGAAGACCCTATAGAGTTTTATATTTATTTATTTTTAAATTTTTAATTAATTTGA
Hyles_e_conspicua         AGACGAGAAGACCCTATAGAGTTTTATATTTATTTATTTTTAAATTTTTAATTAATTTGA
Hyles_hippophaes          AGACGAGAAGACCCTATAGAGTTTTATATTTATTTATTTTTAAATTTTTAATTAATTTGA
Deilephila_elpenor        AGACGAGAAGACCCTATAGAGTTTTATAATTATTAATTTTTAAGTTTTTTATTAATTAAA
Hemaris_fuciformis        AGACGAGAAGACCCTATAGAGTTTTATATATTTATATTTAATTTTTTTATTTTATAGT
Daphnis_nerii             AGACGAGAAGACCCTATAGAGTTTTATAATTAATTATATTTAAAATTTAAATA-AATTGA
Proserpinus_proserpina    AGACGAGAAGACCCTATAGAGTTTGATATTTAATTATTTTAAAGTTTTAAATA-AATAAT
Acherontia_atropos        AGACGAGAAGACCCTATAGAGTTTTATAATTATTTTTTTTTTTATTATAAGTTTATTTAT
Sphinx_pinastri           AGACGAGAAGACCCTATAGAGTTTTATATATTTTTTATTTAAAATTATAAATT--TTATG
Sphinx_ligustri           AGACGAGAAGACCCTATAGAGTTTTATATATTTTTTATTTAAAATTATAAATT-TAAATG
Mimas_tiliae              AGACGAGAAGACCCTATAGAGTTTTATATTTTATAAATTT-AGATTTTAAATA-AATGGT
Smerinthus_ocellata       AGACGAGAAGACCCTATAGAGTTTTATATTTAATTTAATTTAAGTTTTAAATT-TATAGT
Smerinthus_o_atlantica    AGACGAGAAGACCCTATAGAGTTTTATATTTAATTTGATTTAAATTTTAAATT--TATAGT
Laothoe_populi            AGACGAGAAGACCCTATAGAGTTTTATATTTAAATAGATTTAAATTGTAAATTTATTGGT
Laothoe_austauti          AGACGAGAAGACCCTATAGAGTTTTATATTTAAATAGATTTAAATTGTGAATTTATTGGT
Graellsia_isabellae       AGACGAGAAGACCCTATAGAGTTTTATATTATATTGAATTTATTTTATTTATAATTTATT

Manduca_sexta             ATAATATATTAAAAATAAAAATATTTTATTGGGGTGATAAAAAAATTTGTTTAACTTTTT
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Table 15 (Continued)

```
Hyles_livornica          TTATTTTAAATATT?TAAAAATATTTTATTGGGGTGATAAAAAAATAAATAAAACTTTTT
Hyles_euphorbiae         TTATTTTAAATATAGTAAAAATATTTTATTGGGGTGATAAAAAAATAAGTAAAACTTTTT
Hyles_e_conspicua        TTATTTTAAATATAGTAAAAATATTTTATTGGGGTGATAAAAAAATAAGTAAAACTTTTT
Hyles_hippophaes         TTATTTTAAATATAATAAAAATATTTTATTGGGGTGATAAAAAAATAAGTAAAACTTTTT
Deilephila_elpenor       TTAATTTAAAAATTAT-TAATTATTTTATTGGGGTGATAAAAAAATAAATTAAACTTTTT
Hemaris_fuciformis       TTATTTTAGATTAATT-TATATATTTTATTGGGGTGATAAAAAAATNAAATTAACTTTTT
Daphnis_nerii            TTATTTTAAATATTTT-AATATATTTTATTGGGGTGATAGAAAAATAAATTTAACTTTTT
Proserpinus_proserpina   TTATTTTAAAATAAAT-TAATTATTTTGTTGGGGTGATAAAAAAATAAATAAAACTTTTT
Acherontia_atropos       AAACTTAAATAGAAGT-AATATATTTTATTGGGGTGATAGAAAAATTTATTTAACTTTTT
Sphinx_pinastri          ATAAATTTTAATTAGTGGGTATATTTTATTGGGGTGATATAAAAATTTATTTAACTTTTT
Sphinx_ligustri          ATATATTTTAATTAATGGTTATATTTTATTGGGGTGATAGAAAAATTTATTTAACTTTTT
Mimas_tiliae             AATTTAAATTTAAAAA-AGAATATTTTATTGGGGTGATGAAAAAATTTATTTAACTTTTT
Smerinthus_ocellata      AATTAAAATTAATTAA-AAAGTATTTTATTGGGGTGATA-AAAAATTTATTTAACTTTTT
Smerinthus_o_atlantica   AATTAAAATTAATTAA-AAAATATTTTATTGGGGTGATA-AAAAATTTAATTAACTTTTT
Laothoe_populi           AGTTTATATTGATTT--GAATTATTTTGTTGGGGTGATAGGAAAATTGAAATAACTTTTT
Laothoe_austauti         AGTTTATATTTATTT--AAATTATTTTATTGGGGTGATAGAAAAATTGAAATAACTTTTT
Graellsia_isabellae      GAATATAAATTTGATG-TTAATATTTTGTTGGGGTGATAAAAAAATAAAAAAAACTTTTT

Manduca_sexta            -TTAAAATTTTACATAAATAAGTGGTTTATTGATCCAATAATATTGATTATAAGAAAAAA
Hyles_livornica          -TTAATAATAAACATAAATAAATGAATTATTGATCCAAAATTTTTGATTATAAGAAAAAA
Hyles_euphorbiae         -TTAATAATAAACATAAATAAGTGATTTATTGATCCAAAATTTTTGATTATAAGAAAAAA
Hyles_e_conspicua        -TTAATAATAAACATAAATAAGTGATTTATTGATCCAAAATTTTTGATTATAAGAAAAAA
Hyles_hippophaes         -TTAATAATAAACATAAATAAGTGATTTATTGATCCAAAATTTTTGATTATAAGAAAAAA
Deilephila_elpenor       -TTTATATATAACATAAATAAGTGATTAATTGATCCAATATTTTTGATTATAAGAAAAAA
Hemaris_fuciformis       -TTAAATTTAAACATAAATAGGTGATTTTTTGATCCAATAATATTGATTATAAGAAAAAA
Daphnis_nerii            -TTAATTTAAAACATGAATAAGTGATTAATTGATCCAAAATTTTTGATTAAAAGAAAAAA
Proserpinus_proserpina   -TTAATTTTTAACATAAATAAGTGAATTATTGATCCATTATTATTGATTATAAGATAAAA
Acherontia_atropos       -TTAAATTTTAACAAATATAATTGAATTATTGATCCAATATTATTGATTATAAGAAAAAA
Sphinx_pinastri          -TTAAAATTTTACATAAATAAGTGATTTTTTGATCCAATATTATTGATTATAAGAAAAAA
Sphinx_ligustri          -TTTTAAGTTTACATAAATAAGTGATTTTTTGATCCAGTATTATTGATTATAAGAAAAAA
Mimas_tiliae             -ATAAAAAAATCCATAAATAAGTGAATTTTTGATCCAATAATAATGATTATAAGAAAAAA
Smerinthus_ocellata      -TTTAAATTTAACATAAATAAGTGAATTTTTGATCCATTTTTAATGATTATAAGAAAAAA
Smerinthus_o_atlantica   -TTTAAGTTTAACATAAATAAATGAATTTTTGATCCATTTTTAATGATTATAAGAAAAAA
Laothoe_populi           TTAAAATTATACCATAAATAAGTGAATTTTTGATCCAATATTATTGATTATAAGAAAAAA
Laothoe_austauti         TTAAAATTATACCATAAATAAGTGAATTTTTGATCCAATATTATTGATTATAAGAAAAAA
Graellsia_isabellae      -TTAAATTTAACCATAGATAAGTGAATTTTTGATCCAATTATATTGATTATAAGAAAAAA

Manduca_sexta            TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTTAGTACAAATAAGAAAAAAAGTTTGCG
Hyles_livornica          TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAAAAAAGTTTGCG
Hyles_euphorbiae         TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAAAAAAGTTTGCG
Hyles_e_conspicua        TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAAAAAAGTTTGCG
Hyles_hippophaes         TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAAAAAAGTTTGCG
Deilephila_elpenor       TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAATAAAGTTTGCG
Hemaris_fuciformis       TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTACAAATAAAAAAAAGAGTTTGCG
Daphnis_nerii            TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAAAAGAGTTTGCG
Proserpinus_proserpina   TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAAAAAAGTTTGCG
Acherontia_atropos       TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTACAAATAAGAAGAAAAAGTTTGCG
Sphinx_pinastri          TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTACAAATAAAAAAAAAGAGTTTGCG
Sphinx_ligustri          TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTACAAATAAAAAAAAAAAGTTTGCG
Mimas_tiliae             TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTACAAATAAGAAAAAAAGTTTGCG
Smerinthus_ocellata      TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAAAAAGAGTTTGCG
Smerinthus_o_atlantica   TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAGAAGAGTTTGCG
Laothoe_populi           TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAAAAGAGTTTGCG
Laothoe_austauti         TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCAAATAAGAAAAAGAGTTTGCG
Graellsia_isabellae      TTACCTTAGGGATAACAGCGTAATTTTTTTTTTTTAGTTCTTATAAGAAGAAAAGTTTGCG

Manduca_sexta            ACCTCGATGTTGGATTAAGATA
Hyles_livornica          ACCTCGATGTTGGATTAAGATA
Hyles_euphorbiae         ACCTCGATGTTGGATTAAGATA
Hyles_e_conspicua        ACCTCGATGTTGGATTAAGATA
Hyles_hippophaes         ACCTCGATGTTGGATTAAGATA
Deilephila_elpenor       ACCTCGATGTTGGA??AAGA?A
Hemaris_fuciformis       ACCTCGATGTTGGATTAAGATA
Daphnis_nerii            ACCTCGATGTTGGATTAAGATA
Proserpinus_proserpina   ACCTCGATGTTGGATTAAGATA
Acherontia_atropos       ACCTCGATGTTGGATTAAGATA
Sphinx_pinastri          ACCTCGATGTTGGATTAAGATA
Sphinx_ligustri          ACCTCGATGTTGGATTAAGATA
Mimas_tiliae             ACCTCGATGTTGGATTAAGATA
Smerinthus_ocellata      ACCTCGATGTTGGATTAAGATA
Smerinthus_o_atlantica   ACCTCGATGTTGGATTAAGATA
Laothoe_populi           ACCTCGATGTTGGATTAAGATA
Laothoe_austauti         ACCTCGATGTTGGATTAAGATA
Graellsia_isabellae      ACCTCGATGTTGGATTAAGATA
```

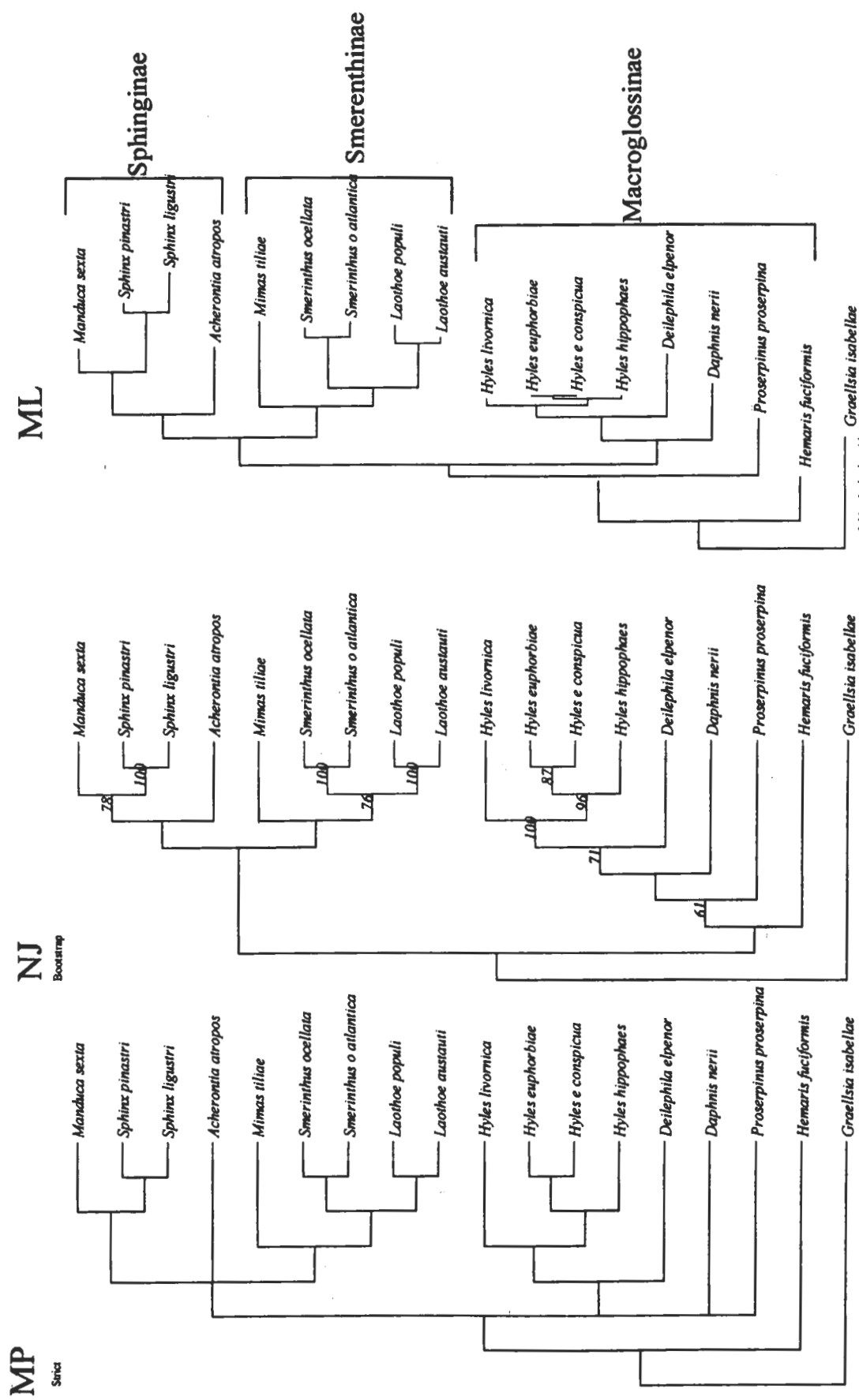

Fig. 2 Molecular phylogeny of the Sphingidae based on a partial sequence of mitochondrial 16S rDNA. Reconstruction with Maximum Parsimony (MP), Neighbor joining (NJ) and Maximum likelihood (ML). *Graellsia isabellae* (Saturniidae) was used as an outgroup. MP analysis: A = 0.391, C = 0.079, G = 0.142; T = 0.388; length 324 steps, CI = 0.506; RI = 0.59; HI = 0.494

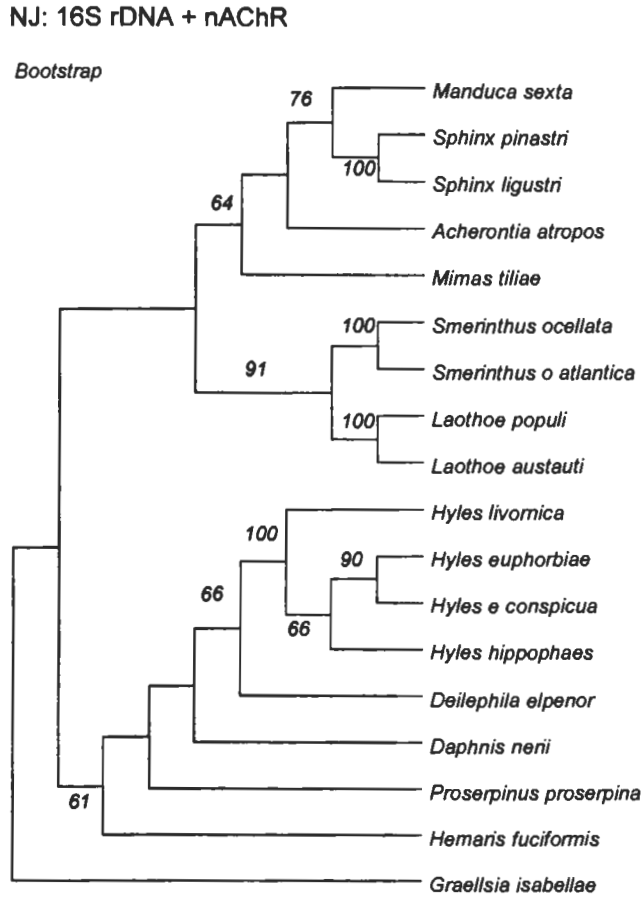

Fig. 3 Phylogenetic tree of Sphingidae based on nucleotide sequences of nuclear $\alpha 1$ and $\alpha 2$ subunits of nAChR and 16S rDNA (combined data set); reconstruction via NJ using Jukes Cantor as a distance algorithm; bootstrap values are based on 1000 replications (only bootstrap values over 60% are shown)

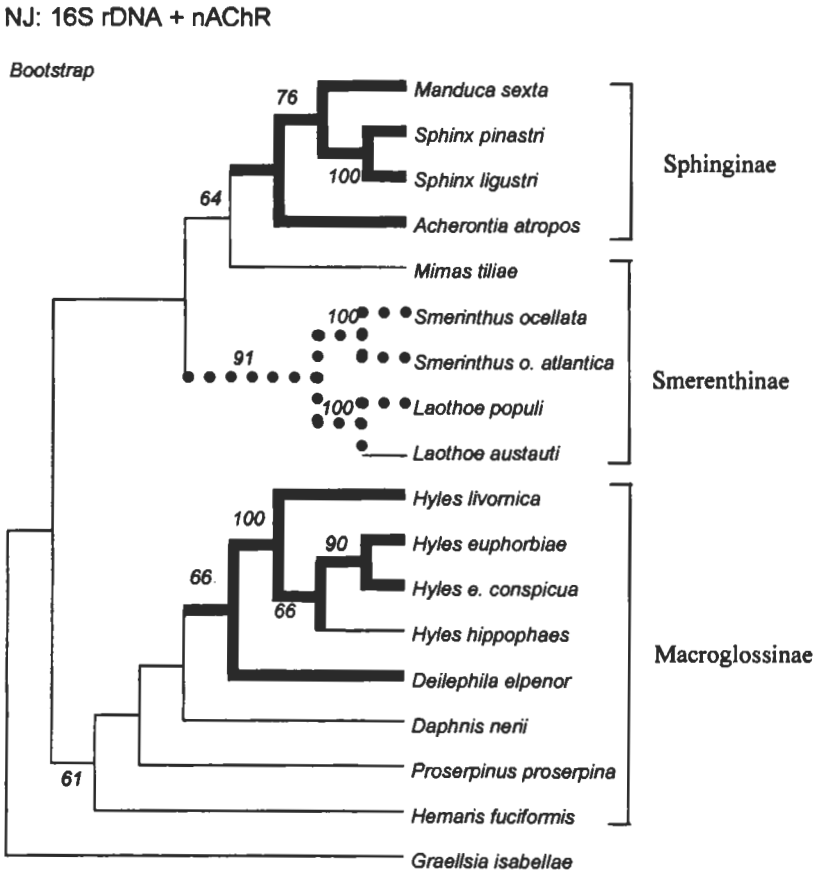

Fig. 4 Evolution of nicotine tolerance in the Sphingidae (data from Fig. 3) Branches in bold: nicotine tolerant species; branches dotted: nicotine sensitive species. Toxicity data were not available for the other taxa

with *Hemaris* at its base (Fig. 2). The monophyly of these tribes is also supported by nucleotide sequences of $\alpha 1$ and $\alpha 2$ subunits of AChR (Tables 11 and 12) combined with 16S rDNA and trees reconstructed from them (Fig. 3). Because of the limited number of species included in this study, the trees obtained must be interpreted with caution and should be regarded as preliminary. However, also another recent study confirmed the sistergroup relationship of Smerenthinae and Sphinginae and the monophyly of Macroglossinae. Also *Hemaris* was found as the ancestral taxon in the Macroglossinae (Regier *et al.* 2001).

We have chosen a total evidence tree (Fig. 3) to map the nicotine toxicity data determined in this study (also the other trees and the morphology data would support the conclusions drawn). As can be seen from Fig. 4, nicotine tolerance appears as a common trait in the Sphinginae and Macroglossinae which feed on toxic and non-arborescent plants, whereas the Smerenthinae feeding on tanniferous trees show a higher degree of nicotine sensitivity. Since we were not able to assess nicotine toxicity in the basal taxa of the Macroglossinae (*Hemaris*, *Proserpinus*, and *Daphnis* nerii) we cannot say whether nicotine tolerance is a plesiomorphic trait of the Sphingidae that was secondarily lost in the Smerenthinae, or instead evolved independently in Sphinginae and Macroglossinae. We need to enlarge the base for toxicity data and phylogeny before we can assess character state evolution in the Sphingidae with confidence.

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