Influence of Food and Larval Age on the Defensive Chemistry of *Saturnia pyri*

Reinhold Deml

Lehrstuhl für Tierökologie II, Universität Bayreuth, D-95440 Bayreuth, Germany.
E-mail: reinhold.deml@uni-bayreuth.de

Z. Naturforsch. **56c,** 89–94 (2001); received September 12/October 18, 2000

Chemical Defense, Saturniidae, Secondary Compounds

Scolus secretions and hemolymph of caterpillars of *Saturnia pyri* fed with two different foodplants (*Crataegus monogyna*, *Prunus spinosa*) were chemically analyzed and their chemical similarities determined. The secondary-compound patterns obtained for the two body fluids showed no significant differences when compared between the two groups of alternatively fed last-instar larvae. Thus, the composition of these fluids of full-grown caterpillars is not influenced by the larval diet. However, younger larvae on *P. spinosa* revealed a diversity of compounds differing significantly from that of larger caterpillars fed with either *C. monogyna* (both body fluids) or *P. spinosa* (hemolymph only). This indicates that, on the one hand, the hemolymph composition is adapted to the changing physiological requirements of the given instars whereas, on the other hand, the defensive mixtures remain unaltered in the late larval instars due to a constant spectrum of potential enemies.

Introduction

Full-grown caterpillars (L5) of the polyphagous Great peacock moth, *Saturnia pyri* (Denis and Schiffermüller) (Lep.: Saturniidae), have a multitude of conspicuous sky-blue colored outgrowths ("scoli") which they bear on their body surface. Haffer (1921) first described the histological changes of these bristle-set structures during larval development and assumed them to be defensive glands. On mechanical irritation, the larvae are able to discharge a clear secretion out of the tips of the black bristles. Later, Deml and Dettner (1993, 1997) showed that the bristles are hollow and that the secretion as well as the hemolymph of larvae fed with *Prunus spinosa* (Rosaceae) contain a variety of secondary compounds, most of which are aromatics. However, while other caterpillars of Saturniidae investigated (e.g., *Attacus atlas*; Deml and Dettner, 1994) deplete a multitude of biogenic and biologically effective amines (e.g., acetylcholine, catecholamines) with their secretions, *S. pyri* and a closely related species, *Eudia pavonia*, seem to discharge mainly phenolic components along with the main compound, glycerol, with their secretions (Deml and Dettner, 1993, 1997).

The question arose where the substances in *S. pyri* might originate from. Pesticides as their potential source could already be exluded (Deml and Dettner, 1997). In order to get more information about the possible origin of the compounds found in *S. pyri*, caterpillars of this species were now reared on either hawthorn (*Crataegus monogyna*; Rosaceae) or sloe (*P. spinosa*), and the glandular secretions and the hemolymph were analyzed by gas chromatography / mass spectrometry (GC/MS). The larvae of *S. pyri* have been reported to feed on many deciduous trees and bushes in the field and in captivity (e.g., Gardiner, 1982; Stone, 1991), and it is most probable that the caterpillars use the two foodplants also in nature (W. A. Nässig, personal communication). Additionally, total ion chromatograms of individual larvae fed with *P. spinosa* were reinvestigated and the respective individual patterns of secondary compounds were compared with each other as well as with the new data. Furthermore, the two body fluids of caterpillars in the third larval instar fed with *P. spinosa* were analyzed in order to find out possible changes in the metabolic patterns during ontogenesis. Finally, the chemical similarities between all the body fluids were computed.

Materials and Methods

Insect material

Living specimens of *S. pyri* were obtained commercially and the larvae (*ex ovo*) were fed with either *C. monogyna* or *P. spinosa* for their whole lives. The animals were reared in aerated plastic

0939–5075/2001/0100–0089 $ 06.00

boxes at 22 °C and 60–70 % relative humidity. During the rearings the number of caterpillars per box was reduced (down to two last-instar larvae, at most) due to the increasing body size of the larvae. Caterpillars in the last and third instar (shortly before molting), respectively, were killed by freezing and stored at −20 °C until used. Secretion was obtained by pressing living caterpillars strongly with the fingers or with forceps, or by defrosting the frozen larvae and pressing the scoli and underlying integument gently with forceps. The clear secretion that flowed out was sucked up with a glass capillary; cloudy secretion droplets would have indicated a contamination with hemolymph in defrosted caterpillars and would not have been used. To sample the hemolymph, a small cut was made with a scalpel in the integument near the prolegs of defrosted larvae, and the hemolymph droplets were sucked up with a glass capillary. Although there is a possibility that such hemolymph from defrosted caterpillars contains intracellular fluid from bursted hemocytes and other cells, at least the glandular cells in the scoli were never observed to have been ruptured and thereby have depleted their secretion into the hemolymph (Deml, unpublished), possibly due to the high titers of glycerol in both, hemolymph and secretion (see "Results and Discussion" section). The respective body fluids were pooled in all cases (five L5 larvae each on *C. monogyna* and *P. spinosa*, four L3 larvae on *P. spinosa*). Because the larvae discharged relatively large amounts of secretion sufficient for the analyses already as third-instars, only four L3 larvae were killed. The remaining, somewhat larger number of five living caterpillars was reared to the last instar in order to be able to compensate for possible losses due to disease, but then could be analyzed as a whole because the larvae staid healthy. The total volume of pooled body fluids per analysis was identical in all cases.

GC/MS

Caterpillar body fluids were chemically analyzed by transferring them from the capillary onto a Solid Injektor SI 1 (SGE) syringe and injecting into a Carlo Erba GC 6000 Vega gas chromatograph containing either a 12.5-m or a 36-m glass capillary column FS – OV 1701 (Chrompack) coupled to a Finnigan-MAT ITD mass spectrometer. The temperature program was 50 °C to 260 °C (10 °C/min). The carrier gas was helium. Electron impact ionization (EI) mass spectra were obtained in total ion chromatograms and compared to mass spectral data of the NBS library and the mass spectra registries of Stenhagen *et al.* (1974) and McLafferty and Stauffer (1989). For confirmation of supposed compounds, authentic chemicals were injected and retention times and mass spectra compared.

Computation of chemical similarity

The chemical data obtained were subjected to clustering algorithms using the computer program NTSYS-PC 1.50 (Applied Biostatistics Inc.). At first, the relative amounts of compounds identified from the larval body fluids were further standardized by division by 100. Then, Euclidean distances (dissimilarity coefficients) were computed from an input rectangular matrix of the values using the SIMINT program. Subsequently, several clustering methods were applied to the resulting dissimilarity matrix: average-based methods such as the unweighted pair-group method using arithmetic averages (UPGMA), as well as the single-link method. Cophenetic correlation coefficients (r_{CS}) were determined through a cophenetic value matrix.

Results and Discussion

Secondary compounds in S. pyri

Evaluation of the total ion chromatograms obtained after injection of body fluids of *S. pyri* reared on different food plants, revealed the presence of a multitude of secondary compounds, in each case (Table I). All of the substances identified as well as their fragmentation behavior are already known from Saturniidae (Deml and Dettner, 1993, 1997, and unpublished results). It shall be noted that both GABA and its lactam, 2-pyrrolidone, give the same mass spectrum due to a decomposition process of GABA (Deml and Dettner, 1995); therefore it could not be decided by GC/MS which of the two compounds actually occur in *S. pyri*. In the course of the evaluations, the hitherto only assumed presence (Deml and Dettner, 1993) of several compounds (phenol, hy-

Table I. Volatile compounds detected by GC/MS of larval body fluids of *Saturnia pyri* fed with either *Prunus spinosa* or *Crataegus monogyna*[1,2].

Larval instar (foodplant) body fluid / **Compound (%)**	L5 (*C. monogyna*) GS	L5 (*C. monogyna*) HL	L3 (*P. spinosa*) GS	L3 (*P. spinosa*) HL	L5 (*P. spinosa*) GS	L5 (*P. spinosa*) HL
Toluene	–	–	–	0.15	–	0.39
o-Xylene	–	–	–	–	–	0.08
Phenol	0.25	0.21	0.88	1.22	–	0.02
o-Cresol	–	–	–	–	0.06	<0.01
p-Cresol (1)	–	–	0.14	0.21	2.85	0.21
3,5-Dimethylphenol	–	–	–	–	2.39	0.51
Hydroquinone	0.31	0.39	1.67	3.35	0.42	–
Veratrole	–	–	–	–	1.20	–
Benzaldehyde	0.34	0.06	4.12	0.61	0.30	0.13
Phenylacetaldehyde	0.16	1.65	2.57	5.60	–	0.36
Benzonitrile (2)	0.31	0.12	1.62	3.22	1.11	0.06
Benzamide (3)	1.09	0.34	2.37	1.95	–	–
Nicotinic acid (7)	–	–	0.23	–	–	–
Nicotinamide	–	0.23	3.73	2.13	–	0.02
2-Pyrrolidone / GABA	1.09	0.61	3.86	6.39	3.05	0.12
N-Methyl-2-pyrrolidone	0.11	–	1.34	3.65	–	0.09
Nicotine (8)	1.02	0.28	–	–	–	–
Pyrazine	0.04	0.09	0.46	1.22	–	0.16
Acetylcholine (4)	–	–	0.39	–	–	–
2-(N,N-dimethylamino)ethyl acetate (5)	–	–	–	4.02	–	–
Choline	–	1.07	1.47	4.26	5.28	–
2-(N,N-dimethylamino)ethanol (6)	0.73	–	15.96	6.08	–	–
Isopropyl myristate (9)	0.23	0.21	–	–	–	–
Glycerol	94.35	94.74	59.19	55.96	83.23	97.88

[1] Percent values given for single components represent their parts in the total peak-area (= 100 %) of all compounds identified per chromatogram obtained after single injection of pooled body fluids. L3/L5 = third/fifth larval instar; GS = glandular secretion; HL = hemolymph; – = not detected. Numbers in parentheses refer to Fig. 1.

[2] Statistical significancies:
L5 (*C.m.*) GS – L5 (*P.s.*) GS: **n.s.** L3 (*P.s.*) HL – L5 (*P.s.*) HL: **
L5 (*C.m.*) HL – L5 (*P.s.*) HL: **n.s.** L3 (*P.s.*) HL – L5 (*C.m.*) HL: *
L3 (*P.s.*) GS – L5 (*P.s.*) GS: **n.s.** L3 (*P.s.*) GS – L5 (*C.m.*) GS: *
(Wilcoxon-Mann-Whitney U-test, two-tailed case; actually used level of significance was corrected according to Bonferroni's method to $\alpha/6$; ** = $0.01 \geq P > 0.001$; * = $0.05 \geq P > 0.01$; **n.s.** = not significant).

droquinone, benzaldehyde) in secretion and/or hemolymph of L5-larvae on *P. spinosa* could be ascertained whereas another uncertain component from previous analyses, 2-aminoethyl acetate, proved to be missing in these animals in detectable amounts. Additionally, several compounds were identified for the first time from samples of *S. pyri* (Fig. 1). For example, benzonitrile (**2** in Fig. 1) could be detected in hemolymph and glandular secretion of these caterpillars. The biogenic amine, acetylcholine (**4** in Fig. 1), was previously known to occur only in the gland secretions of the saturniids, *Eupackardia calleta* and *Attacus atlas*; now it could be found also in the secretion of the L3-larvae of *S. pyri* fed with *P. spinosa* but not in the corresponding last-instar larvae. A particularly interesting finding is that benzoic acid amide (**3** in Fig. 1) has been identified from L5 larvae fed with *C. monogyna* and from L3 larvae fed with *P. spinosa* (as well as recently from larvae of the saturniid, *Hyalophora cecropia*; Deml and Dettner, unpublished results). This chemical was assumed to be a precursor of the unusual compound, benzonitrile, in Saturniidae. However, because benzamide could not be found in the saturniid caterpillars previously investigated, including in L5 larvae of *S. pyri* on *P. spinosa* (Deml and Dettner, 1993; see also Table I), the amino acid, phenylglycine, which was identified instead was at that time assumed to be the only potential precursor.

Influence of larval food

When L5 caterpillars fed with the two different foodplants are compared, no significant differences can be stated ($P > 0.05$; Wilcoxon-Mann-Whitney U-test, two-tailed case; actually used level of significance was corrected according to

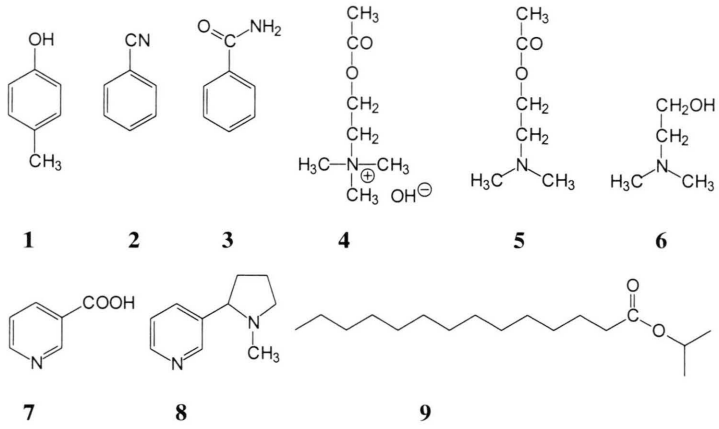

Fig. 1. Newly detected compounds in hemolymph and/or defensive secretions of *Saturnia pyri*. **1** = *p*-cresol, **2** = benzonitrile, **3** = benzamide, **4** = acetylcholine, **5** = 2-(N,N-dimethylamino)ethyl acetate, **6** = 2-(N,N-dimethylamino)ethanol, **7** = nicotinic acid, **8** = nicotine, **9** = isopropyl myristate.

Bonferroni's method to $\alpha/6$). In Table I, glandular secretion of L5 larvae fed with *Crataegus* contains more compounds than that of *Prunus*-eaters while the reverse is true of the respective hemolymphs, and either of the two groups of caterpillars also contains substances exclusive to the corresponding foodplant; however, these differences cannot be statistically corroborated and therefore must not be regarded as being appreciable. A similar comparison of two groups of larvae of *Eudia pavonia* from an identical feeding trial revealed an even better chemical correspondence of the respective body fluids (Deml and Dettner, 1993) whereas the secondary chemistry of *E. pavonia* was distinctly different from that of the related species, *S. pyri*. In view of partially considerable individual variations now determined for four L5 larvae of *S. pyri* fed with *P. spinosa* (standard deviations as large as the means in the case of several trace compounds such as cresol, 3,5-dimethylphenol, benzaldehyde, benzonitrile; see also Deml and Dettner, 1993), it cannot be excluded that the respective secretions and hemolymphs will become chemically even more similar when a larger sample size of caterpillars is analyzed. Important individual variations (differing quantities of compounds, even absence of substances, especially of trace constituents) have been established also in the trial with larvae of *E. pavonia* fed with either of the two plants.

In the course of chemical investigations of the two plants by GC/MS using various assays (crude extracts as well as different methods of cleavage of glycosides and other conjugates), from the low-molecular caterpillar-compounds identified, only minor amounts of benzaldehyde and nicotinic acid were detected, and only in *P. spinosa* (Deml and Dettner, 1997 and unpublished results). Typical constituents of the two foodplants known from literature (mainly glycosides and their aglyca, respectively, several of which are not detectable by GC/MS; e.g., Gessner and Orzechowski, 1974; Hegnauer, 1962–1996; Hunnius *et al.*, 1998) could not be identified from the larval body fluids. Typical constituents of *P. spinosa* are prunasin/amygdalin/mandelonitrile, prunitrin/prunetin, genistein, rutin, trifolin, quercitrin/quercetin, kaempferol, many other flavonoids, catechines and leucocyanidins, scopoletin, *p*-coumaric acid, sitosterol, and free amino acids. *C. monogyna* contains, for example, prunasin/amygdalin/mandelonitrile, oligo- and monomeric procyanidins and catechines, flavonol glycosides (e.g., hyperin, rutin, quercitrin/quercetin, vitexin, vitexin rhamnoside and a monoacetate thereof, other vitexin glycosides), esculin/esculetin, veratric, crataegic, crataegolic, oleanolic, ursolic, and chlorogenic acids, sitosterols, and amines such as trimethylamine and tyramine. Reversely, a few of these plant compounds although being present in the caterpillars were not detected from fresh material of the plants used as a food (*C. monogyna*: choline and acetylcholine). It seems that most of the detectable, described plant-substances are excreted or converted quantitatively into other secondary compounds preferred by the caterpillars; for example, prunasin/mandelonitrile could be metabolized to benzaldehyde and phenylacetaldehyde. Such an occasional utilization of specific plant-components could explain the individual variability of the larval chemistry established

which is oftenly observed in insects which defend themselves chemically and ingest components from foodplants (Whitman, 1988).

However, the present result that there is no difference between the chemistry of the two groups of larvae supports the assumption that most of the substances are biosynthesized by the caterpillars themselves (Deml and Dettner, 1993). It may be assumed that certain polar substances which are generally and reliably contained in appropriate foodplants are used as favored starting-points of the synthesis of defensive compounds by the caterpillars. For example, the aromatic amino acids (tyrosine and phenylalanine) or other aromatic acids such as benzoic or cinnamic acid and their derivatives could be suitable precursors of most larval aromatics (Deml and Dettner, 1993, 1997). Many peaks whose mass spectra corresponded to derivatives of cinnamic acid and benzoic acid according to the NBS spectra library, could be detected in assays of the two plants by GC/MS (Deml and Dettner, unpublished results).

Influence of larval age

Striking differences can be found on comparison of L3- and L5-larvae of *S. pyri* (Table I). The younger caterpillars fed with *P. spinosa* contain a larger diversity of secondary compounds than the two groups of full-grown larvae, resulting in statistically significant differences of L3 and L5 hemolymph on *P. spinosa* ($0.01 \geq P > 0.001$; Wilcoxon-Mann-Whitney U-test, two-tailed case; actually used level of significance was corrected according to Bonferroni's method to $\alpha/6$), L3 and L5 hemolymph on *Crataegus*, as well as L3 and L5 secretion on *Crataegus* (both tests: $0.05 \geq P > 0.01$), while L5 secretion on *Prunus* cannot be statistically separated from L3 secretion on the same plant (nominal $P = 0.0875$). Although there are large differences for some compounds implied in the last-mentioned comparison (e.g., 2-(N,N-dimethylamino)ethanol, benzaldehyde, nicotinamide), the two secretions as a whole must be considered equal and containing random outliers, taking the statistical result into account.

Clustering of the different glandular secretions and hemolymphs by means of average-based methods (e.g., UPGMA; Fig. 2) as well as by single-linkage (data not shown; $r_{CS}=0.97$) resulted in

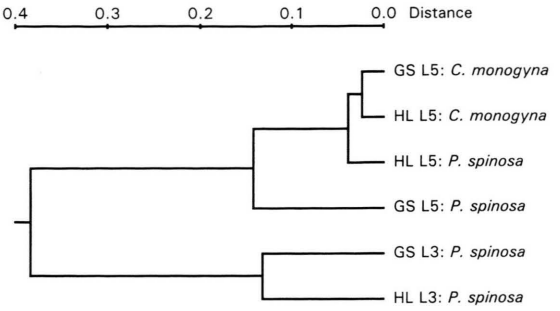

Fig. 2. Phenogram of gland secretions (GS) and hemolymph (HL) from caterpillars of *Saturnia pyri* fed with either *Prunus spinosa* or *Crataegus monogyna*. L3/L5 = third/fifth larval instar. Euclidean distances (dissimilarity coefficients) were computed from data matrix produced from relative amounts of 24 secondary compounds in pooled body fluids (Table I). Clustering by UPGMA; $r_{CS} = 0.97$.

the formation of two distinctly separated groups: one cluster of the L3 larvae on *P. spinosa* and another one of the L5 caterpillars, irrespective of their food. This indicates that the L5 larvae are chemically more similar to each other than to L3 larvae.

Consistently, the influence of larval food is much less crucial for the secondary compound patterns of the caterpillars than their age. The hemolymph composition changes during growth of the caterpillars what might be caused mainly by physiological needs and alterations of the larvae. The relative amounts of substances such as phenol, hydroquinone, and choline are reduced in hemolymph during the larval development; 2-(N,N-dimethylamino)ethyl acetate even completely disappears. Simultaneously compounds such as glycerol (serves as a solvent and anti-freezing agent and assists other compounds to penetrate the skin of enemies; Deml and Dettner, 1993, 1994) and nicotinamide in hemolymph as well as benzonitrile in scolus secretions are synthesized more strongly or they are possibly transferred from the hemolymph into the secretions (benzonitrile) or vice versa (nicotinamide).

In case of the exocrine secretions as a whole, the relative amounts of components remain unchanged on the same foodplant in a statistical respect. This indicates that the caterpillars have no need to produce a distinctly altered bouquet of secondary compounds in order to prevent themselves from a spectrum of "target enemies" of the

defensive fluids (e.g., ants, birds) which is obviously not changing substantially in the course of the late larval development. In contrast to this, the chemical composition of the osmeterial secretions of several swallowtails (*Papilio* spp.; Lep.: Papilionidae) changes distinctly from the fourth larval instar (mainly terpenes) towards the fifth instar (primarily aliphatic acids and their esters; Honda, 1981) while no significant influence of foodplant chemistry on the constitution of the *de novo* synthesized secretions has been detected (in fourth instar larvae; Honda, 1983). Although this chemical change coincides with a striking change of larval coloration (L4: bird droppings, i.e., cryptic; L5: eyespots, i.e., aposematic at short distances), no different palatability of L4 and L5 larvae to birds could be demonstrated (Leslie and Berenbaum, 1990); rather, the ontogenetic variation of osmeterial secretions could be attended with their supposed function as an olfactory warning signal or with defense against differing invertebrate predators. Whether the defensive chemistry of *S. pyri* changes on comparison of younger larvae (L1, L2, early L3) with larger ones remains to be investigated but appears possible in view of several changes of scolus-colors observed during larval development. In the present paper, significantly different compositions of the defensive secretions of *S. pyri* can be found only if extremes are compared (L3 on *P. spinosa* – L5 on *C. monogyna*). Only in such a case an influence of the larval diet can be manifested but is probably of minor biological significance and, rather, of statistical interest. However, now as before, only controlled feeding studies using tracer compounds, and observations of cases of larval defense in the field can probably demonstrate the validity of these assumptions ultimately.

Acknowledgements

I thank Prof. Dr. K. Dettner, Universität Bayreuth, and Prof. Dr. K. Fiedler, Universität Bayreuth, for critical comments and remarks on statistics. I am also grateful to an anonymous reviewer for helpful improvements of the manuscript.

Deml R. and Dettner K. (1993), Biogenic amines and phenolics characterize the defensive secretion of saturniid caterpillars (Lepidoptera: Saturniidae): a comparative study. J. Comp. Physiol. B **163,** 123–132.

Deml R. and Dettner K. (1994), *Attacus atlas* caterpillars (Lep., Saturniidae) spray an irritant secretion from defensive glands. J. Chem. Ecol. **20,** 2127–2138.

Deml R. and Dettner K. (1995), Extrusible glands and volatile components of the larval gypsy moth and other tussock moths (Lepidoptera: Lymantriidae). Entomol. Gener. **19,** 239–252.

Deml R. and Dettner K. (1997), Chemical defence of emperor moths and tussock moths (Lepidoptera: Saturniidae, Lymantriidae). Entomol. Gener. **21,** 225–251.

Gardiner B. O. C. (1982), A silkmoth rearer's handbook. The Amateur Entomologist **12**, 3rd edition. The Amateur Entomologists' Society, Hanworth, Middlesex.

Gessner O. and Orzechowski G. (1974), Gift- und Arzneipflanzen von Mitteleuropa, 3rd edition. Universitätsverlag C. Winter, Heidelberg.

Haffer O. (1921), Bau und Funktion der Sternwarzen von *Saturnia pyri* Schiff. und die Haarentwicklung der Saturnidenraupen. Ein Beitrag zu dem Thema: Das Arthropodenhaar. Arch. Naturgesch., Abt. A **87,** 110–166.

Hegnauer R. (1962–1996), Chemotaxonomie der Pflanzen. Birkhäuser, Basel.

Honda K. (1981), Larval osmeterial secretions of the swallowtails (*Papilio*). J. Chem. Ecol. **7**, 1089–1113.

Honda K. (1983), Evidence for *de novo* biosynthesis of osmeterial secretions in young larvae of the swallowtail butterflies (*Papilio*): deuterium incorporation *in vivo* into sesquiterpene hydrocarbons as revealed by mass spectrometry. Insect Sci. Appl. **4,** 255–261.

Hunnius C., Burger A. and Wachter H. (1998), Pharmazeutisches Wörterbuch, 8th edition. DeGruyter, Berlin.

Leslie A. J. and Berenbaum M. R. (1990), Role of the osmeterial gland in swallowtail larvae (Papilionidae) in defense against an avian predator. J. Lepid. Soc. **14,** 245–251.

McLafferty F. W. and Stauffer D. B. (1989), The Wiley/NBS Registry of Mass Spectral Data. Wiley, New York.

Stenhagen E., Abrahamsson S. and McLafferty F. W. (1974), Registry of Mass Spectral Data. Wiley, New York.

Stone S. E. (1991), Foodplants of world Saturniidae. Mem. Lepid. Soc. **4**.

Whitman D. W. (1988), Allelochemical interactions among plants, herbivores, and their predators. In: Novel Aspects of Insect-Plant Interactions (P. Barbosa and D. K. Letourneau, ed.). Wiley-Interscience, New York, pp. 11–64.