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Revisiting the organohalogens associated with 1979-samples of Brazilian bees (*Eufriesea purpurata*)

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Abstract

Brazilian bees of the species *Eufriesea purpurata* are known to tolerate very high concentrations of DDT. As reported in the literature, these bees have suffered no harm from as much as 2 mg/bee, which is in the per-cent range of the body weight. In 1979, individuals of *E. purpurata* were captured as they collected DDT from walls of remote, rural houses in Brazil. Reported herein are quantities and identities of DDT, DDT metabolites, and other organohalogen compounds in four samples of bees stored since 1979. The concentrations of DDT (sum of *p,p′*-DDT, -DDE, and -DDD) ranged from 23 to 314 μg/bee which is up to twelve fold higher than the LD_{50} value of DDT in the honey bee (*Apis mellifera*) but significantly lower than the no-effect concentration in *E. purpurata*. Enantioselective determination confirmed the presence of racemic *o,p′*-DDT in the four individual samples. GC/ECNI-MS investigation resulted in the detection of low amounts (<1 μg/bee) of PCA, lindane, and chlordane. At higher retention times four unknown compounds were detected with a proposed molecular ion at *m/z* 498, a non-aromatic hydrocarbon backbone along with the presence of eight chlorine substituents. Neither the structure nor the origin of these compounds could be determined. Considering where and when the bees were collected and considering the biology and ecology of the euglossine bees themselves, we propose that the four unknowns are natural products and, as such, are the most highly chlorinated natural compounds yet discovered.

Keywords: Persistent organic pollutants; Dichlorodiphenyltrichloroethane; Bees; Halogenated natural products

1. Introduction

Several organohalogen compounds that are toxic to insects have been used to control agricultural pests. Used in this way these insecticides also pose a risk to useful insects, such as honey bees. For this reason some pesticides could not be applied during the flowering of plants, or producers would recommend spraying early in the morning or late in the evening to prevent harm to the day-active bees. There are, however, examples of organochlorine insecticides which were relatively non-toxic to bees. For instance, the strong pesticide toxaphene was found to be only moderately toxic to bees. This might have been the clue for the acronym of the former East-German brandmark Melipax ("Meli" most likely stands for the zoological name of the honey bee *Apis mellifera* and "pax" for lat. = peace, i.e. peaceful for bees) (Vetter et al., 1997).

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DDT is another and better known organochlorine compound that is classified as being relatively non-toxic to honey bees. For *A. mellifera* LD_{50} of 12–27 μg/bee has been proposed (Anonymous, 1989; Atkins et al., 1973). Current data on hexachlorocyclohexane (or HCH) and DDT residues in Polish bees were up to 51 and 76.3 ng/g solid matter, respectively, in destructor females (Romaniuk et al., 2004) whereas *A. mellifera* from India contained 85 and 0.6 ng/g (Khan et al., 2004). These concentrations are far below the LD_{50} value. In contrast to *A. mellifera*, the males of a Brazilian species of euglossine bees are actually attracted to DDT (Roberts et al., 1982). The proclivity of euglossines for many aromatics from both floral and non-floral sources suggests these bees could be an interesting model for studies of aromatics and natural organohalogens (Cameron, 2004; Whitten et al., 1993).

The Euglossini are New World solitary bees. The females collect pollen and nectar, and are important pollinators of many flowering plants (Dressler, 1982). It is noteworthy that the females also collect plant resins for constructing nest chambers and Armbruster (1984) proposed they use plant derived chemicals for protecting their broods from pathogens and parasites. Male euglossines also are important pollinators of orchids and other flowering plants. Their role as plant pollinators evolved at some point in the evolution of behavior for visiting non-floral and floral sites to collect chemical aromatics (Cameron, 2004). Examples of non-floral sites are rotting logs, decomposing forest litter, tree wounds, etc. The males use tarsal brushes for collecting chemicals. The chemicals are then passed to the hind legs for storage in internal pouches of the tibia (tibial pouches or tibial organs) (Dressler, 1982). The bees have large lipid-containing labial glands and they use the nonpolar secretions from such glands to increase "absorption efficiency by the tarsal brushes and storage in the tibial organ" (Cameron, 2004).

The morphology and histology of the tibial organ have been described (Cruz Landim et al., 1965). It is commonly referred to as a spongy sack located on the tibia of the hind leg in close relationship with a hairy slit (portal for entry of chemicals). The sack has three zones of cells, with the third zone containing large numbers of mitochondria. The term "mitochondrial pump" has been used to describe the third zone and Cruz Landim et al. (1965) suggest that the bees might chemically modify fragrance stored in the tibial organ. Dressler (1982) suggests there is no consensus that this happens.

Visits of male euglossine bees to rotting logs and other non-floral sites bring males into contact with chemicals produced by the wood rot fungi. These fungi are now known to produce prodigious quantities of organohalogens, and organochlorines in particular (Verhagen et al., 1998; Watling and Harper, 1998).

As mentioned above, males of one euglossine species, *Eufriesea purpurata*, are attracted to DDT (Roberts et al., 1982). House walls in malaria endemic areas of Brazil were sprayed with DDT for malaria control in the 1970s. The bees would enter those houses and scrape DDT from the walls. The bees included in the present report were collected as they harvested the DDT from walls in a remote site of the Amazon Basin in 1979. Subsequent studies showed that the males collected large quantities of DDT and were not harmed by the toxic agent. Concentrations as high as 2000 μg/bee or 42 ppm (mean bee weight ~ 50 mg) were found in the bees (Roberts et al., 1982).

Much is being learned about the role and abundance of organohalogens in the environment and in living organisms. This example of *E. purpurata* harvesting and using DDT for its own purposes further demonstrates the complexity of organohalogens in the chemistry of life. It also shows how even manmade chemicals can fit into a natural chemical process. *E. purpurata*, as with other species of the tribe Euglossini, is a fascinating model for organohalogen studies. The males dedicate very significant portions of their life's activities to collecting chemicals. The reasons they collect and the uses they make of DDT and other organohalogens are unanswered questions (Cameron, 2004; Dressler, 1982). However, their pronounced behavior of collecting DDT leads to the intriguing question of whether there are other natural organochlorine compounds associated with these bees? To answer this question, four bees from 1979 collections were subjected to careful analyses for known and unknown organohalogens.

2. Materials and methods

2.1. Samples

The males of *E. purpurata* were collected at a site known as Floresta along the Ituxi River in the southern part of Amazonas State in Brazil. The collections occurred during the period of September to October, 1979.

Floresta is an extremely remote site, accessible only by boat, and located about 18 h by boat from the town of Labrea. In 1979 there were two families living at Floresta. Nearest neighbors were several kilometers away. The houses were located along the river and closely encircled

by deciduous evergreen tropical forest. The local inhabitants extracted a living from subsistence crops, fishing, and hunting. Other than the DDT sprayed on house walls, the residents had no ready access to commercial insecticides or herbicides, or even commercial chemicals that might be used for cleaning around the house.

The houses were constructed of locally obtained forest products. The walls and floors were constructed of palm slats and the roof was constructed of palm fronds (thatch). At the time bees were collected, we knew of no other insecticides that were available to the local residents. However the houses had been at Floresta for many years so we cannot exclude some historical uses of other insecticides.

Other than their being lured to enter houses to collect DDT, the males of *E. purpurata* had no known association with the domestic environment or human habitations. So, what we are describing here is a setting in which the bees' access to manmade chemicals was severely restricted. However, we cannot entirely exclude exposure to such chemicals.

The bees were collected in 1979 and were killed by vapors of ethyl acetate. Preservation was carried out by drying. The bees might have been exposed to vapors from moth balls (naphthalene) during early years of preservation in museum cabinets. Although unlikely, it is remotely possible that the bees could have been exposed to vapors from Vapona strips (dichlorfos) that might have been used in the 1980s. For this reason, we analyzed control samples. None of the associated samples gave evidence for contamination with organohalogen compounds during the long period of storage.

2.2. Standards

Reference standards of *p,p′*-DDT and related compounds, as well as other chloropesticides or PCBs were obtained from LGC Promochem (Wesel, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). Solvents and other chemicals were of qualities suitable for trace analysis.

2.3. Clean-up procedure

Whole individual bees were cut with a lab-knife and weighed into 250 mL-quartz flasks. Solvent and internal standard were added, and the mixture was extracted by focused-open vessel microwave assisted extraction using a Soxwave 100 system (Prolabo, Paris, France) according to the method of Weichbrodt et al. (2000). Following that, the extract was subsequently concentrated to <2 mL by rotary evaporation and blowing down with nitrogen. Then the solvent was changed to isooctane (final volume 1–2 mL). This isooctane extract was purified on 3 g silica (deactivated with 30% water, w/w) slurry packed in a 1 cm-i.d. glass column (Weichbrodt et al., 2000). The sample was eluted with 60 mL *n*-hexane and was finally concentrated to 0.2 mL (major solutions). Dilutions by 1:100 to 1:500 were prepared for quantification of DDT and metabolites. Undiluted samples were used for the determination of the enantiomer faction (EF) of *o,p′*-DDT by enantioselective GC/EI-MS, and the sample with the highest contamination level was used for GC/ECNI-MS studies on known and unknown compounds. Additionally, one bee sample (bee #3) extract was fractionated in the following manner: 0.05 mL of the major solution was chromatographed on 8 g activated silica using 48 mL of *n*-hexane (fraction 1) and then 50 mL of *n*-hexane/ethyl acetate (1:1; v/v) (fraction 2) (Weichbrodt et al., 2000).

2.4. Gas chromatographic determinations

Quantification of DDT-related compounds was carried out by GC/ECD using a dual column HP 5890 series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) with the parameters previously described in detail (Weichbrodt et al., 2000). Confirmation of known and identification of unknown organohalogen compounds were carried out with a HP5989 MS engine using parameters described elsewhere (Krock et al., 1996). The GC capillary column was a 30 m × 0.25 mm i.d. HP-5 (Krock et al., 1996). In the full scan mode *m/z* 33–*m/z* 650 was recorded throughout the run. Enantioseparation of *o,p′*-DDT was performed with a Chirasil-Dex column (25 m × 0.25 mm i.d.; Varian Chrompack, Middelburg, The Netherlands) installed in a Hewlett-Packard 5971 MSD (Krock et al., 1996). The enantiomers of *o,p′*-DDT were eluted at 145 °C (isothermal). Throughout the run we monitored *m/z* 235 and *m/z* 237.

3. Results and discussion

As anticipated, DDT concentrations in the bee samples were very high (Table 1). The major compound in technical DDT mixtures, *p,p′*-DDT, was dominating but *p,p′*-DDE, *p,p′*-DDD, and *o,p′*-DDT were identified as well (for structures see Fig. 1). Compared to the technical mixture, contribution of *p,p′*-DDD was significantly higher in bees (see footnote in Table 1). This indicates slight transformation, which might as well have occurred during the long period since sampling. To further investigate some potential transformation

Table 1
Concentrations (μg/bee) of *p,p′*-DDT, -DDE, -DDD, and EF of *o,p′*-DDT in four individual bees collected in 1979 in Brazil

	Technical DDT (contribution)	Bee #1 (93 mg)[a]	Bee #2 (108 mg)[a]	Bee #3 (97 mg)[a]	Bee #4 (109 mg)[a]	Mean value
p,p′-DDT	94.7%	17	246	43	40	87
p,p′-DDE	0.4%	3.2	27	4.5	3.9	10
p,p′-DDD	4.9%	2.7	41	7.6	5.8	14
SumDDT[b]		23	314	56	50	110
$EF_{(o,p'\text{-DDT})}$	0.500±0.005	0.502	0.497	0.505	0.497	0.500

p,p′-DDT/-DDE/-DDD ratio in bee #1: 78.5%/12.9%/8.8%.
[a] Dry weight of the bee.
[b] Sum of *p,p′*-DDT, -DDE, and -DDD.

by the bees, we studied for the first time the enantioselective fate of a POP, namely *o,p′*-DDT, in bees. In contrast to the *p,p′*-isomer, *o,p′*-DDT is chiral, and enantioseparation of this compound has recently become a valuable tool for investigating the environmental fate of pollutants (Vetter and Schurig, 1997; Kallenborn and Hühnerfuss, 2001). The samples of bees, however, contained both enantiomers in equal amounts (in racemic ratio). Therefore, enantioselective transformation of *o,p′*-DDT could not be identified (Fig. 2).

The data in Table 1 demonstrates that bee #2 accumulated the highest concentration of DDT. The DDT concentrations in the bees exceeded the LD_{50} value of *A. mellifera* (see above) but were much lower than the no-lethal effect level in *E. purpurata* (Roberts et al., 1982). Bee #2 was used to screen for other halogenated compounds by GC/ECNI-MS. Extraction of *m/z* 35 from the full scan run allows identifying of virtually all organochlorine compounds (Fig. 3). In addition, *m/z* 79 and *m/z* 81 easily identify brominated compounds. However, brominated compounds were not detected in the samples. The investigation of other chlorinated compounds was complicated by the high concentration of DDT-related compounds in the samples. However, several other organochlorines were identified on the basis of their retention times and mass spectra. These compounds include hexachlorobenzene (8.73 min), pentachloroanisole (PCA) (8.90 min, Fig. 3), lindane (9.84 min), the DDT metabolite *p,p′*-DDMU (18.04 min, Fig. 3), and some chlordane-related compounds (18.84, 19.51, 19.85, and 20.13 min). Compared to these chloropesticides the concentrations of chlorinated industrial chemicals were low. Only a few, very low abundant PCB congeners were determined in the sample (<0.1% of the major DDT compounds). The amounts detected were in the range of those of the sample blanks.

The most interesting compounds next to DDT and related compounds eluted between 39 and 42 min (Fig. 3). Four compounds (labeled A–D in Fig. 3) were identified which appeared to belong to the same compound class due to related mass spectra. Compounds A, B and C showed the highest mass at *m/z* 498

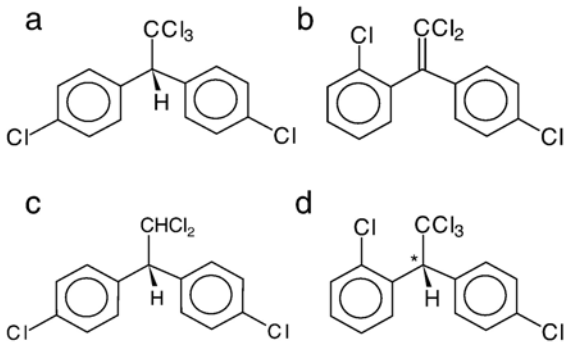

Fig. 1. Structure of (a) *p,p′*-DDT, (b) *o,p′*-DDE, (c) *p,p′*-DDD, and (d) *o,p′*-DDT.

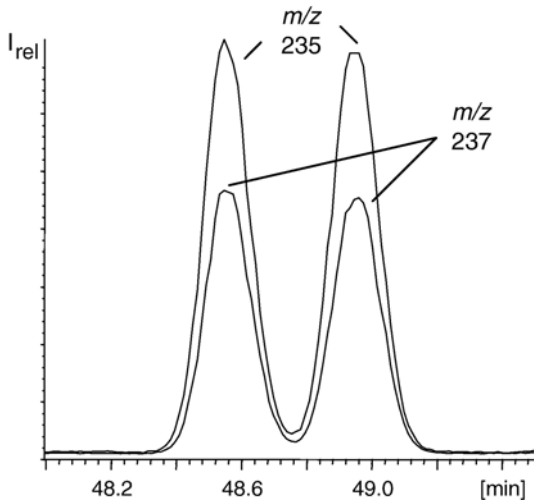

Fig. 2. GC/EI-MS chromatogram (*m/z* 235 and *m/z* 237; Chirasil-Dex) of the enantioselective determination of *o,p′*-DDT in *Eufriesea purpurata.*

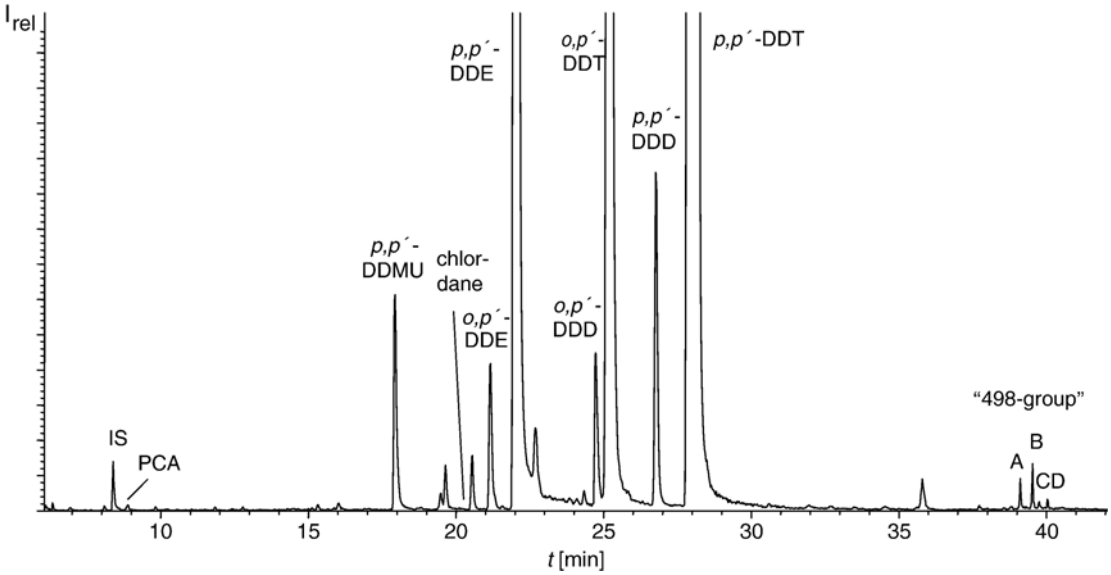

Fig. 3. GC/ECNI-MS chromatogram (*m/z* 35; HP-5) of the organochlorine fraction in *Eufriesea purpurata*.

(Fig. 4). This potential molecular ion was dominated by *m/z* 502, where the distance of isotopic peaks is in agreement with the presence of multiple halogens. Since presence of bromine was excluded (see above), compounds A–D appear to be exclusively chlorinated. The intense fragmentation (Fig. 4) along with the significant abundance of $[HCl_2]^-$ relative to the ions corresponding with $[Cl_2]^-$ indicated a non-aromatic hydrocarbon backbone for compounds A–D. However, aliphatic compounds often do not exhibit the molecular ion in GC/ECNI-MS, which leaves some open questions with regard to the origin of compounds A–D. Furthermore, the chlorine isotope pattern suggested the presence of eight Cl substituents, although the pattern was not in perfect shape (Table 2). No evidence was found for the presence of additional hetero atoms that have a significant impact on the isotopic distribution (i.e. those having isotopic peaks separated by 2 u). In subsequent analyses, the abundances of major ions of the respective isotope pattern were determined in the SIM mode.

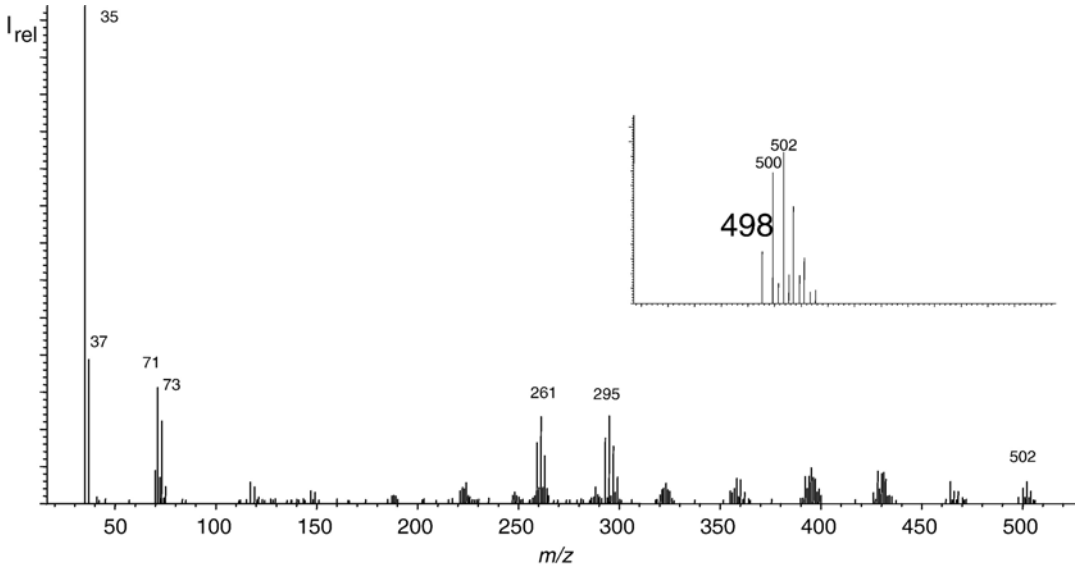

Fig. 4. Mass spectrum of the unknown peak labeled "B" in Fig. 2 as well as the potential molecular ion with the same mass of peak "C".

Table 2
Ratio of the isotopic peaks of octachloro derivatives and the respective ion distribution in the unknown peaks A–D with the proposed molecular ion at *m/z* 498

	498	500	502	504	506	508	510	512	514
Theory [a]	34.93	89.35	100	63.91	25.56	6.54	1.04	0.09	0.0035
Bee #2 [b]	16.5	95.8	100	58.5	21.6	5.4	0.9		
Bee #2, 10 fold diluted [b]	16.5	92.3	100	61.3	19.7	7.4			

[a] Calculated from the theoretical ratio $^{35}Cl/^{37}Cl = 1:0.31978$.
[b] Measured values for the peak at 39.76 min (compound D).

Compounds B and C were used to estimate the number of carbons on these molecules. Determination of the ratio of *m/z* 501/500 and *m/z* 503/502 resulted in ratios of 0.184–0.21 which corresponds with 16.7–19.1 carbons with a typical precision of ±2 carbons. Subtraction of the contribution of chlorine (8 × 35 u) from the molecular ion (498 u–280 u) leaves 218 u for other elements. This allows a maximum of seventeen carbons ($C_{18}H_2Cl_8$ is not possible from a theoretical point of view). Based on these considerations, the following elemental compositions were constructed: (i) $C_{17}H_{14}Cl_8$; (ii) $C_{16}H_{26}Cl_8$, (iii) $C_{16}H_{10}Cl_8O$, (iv) $C_{15}H_6Cl_8O_2$, and (v) $C_{15}H_{10}Cl_8N_2$. Option (ii) corresponds with a saturated aliphatic compound. For verification, we used column chromatography with activated silica where aromatic organohalogens (e.g. PCBs, HCB) elute in fraction 1 and non-aromatic organochlorine compounds (HCH, toxaphene, chlordane) are found in fraction 2 (see Materials and methods for the procedure). In agreement with our predictions, the unknown compounds A–D eluted into fraction 2.

All properties described so far are in agreement with the known contaminants chloroparaffins (Muir et al., 2000). However, there are two remarkable differences which vote against this. First, technical products of chloroparaffins are mixtures of >>100 compounds. In view of the low metabolizing capacity of bees observed in the case of DDT, it is very unlikely that technical chloroparaffins can be the source for the peaks detected in the bees. Second, GC/ECNI-MS spectra of (known) chloroparaffins are dominated by the $[M–Cl]^-$ fragment ion, which is not the case with compounds A–D. Thus, the respective source was excluded.

Two typical fragment ions dominated by *m/z* 261 and *m/z* 293 were found for compounds A through D (Fig. 4). Despite these similarities, the fragmentation of compounds A–D was different. For instance, compound D did not display the potential molecular ion at *m/z* 502. The concentrations of the unknown compounds could only be estimated on the basis of the GC/ECNI-MS response. This led to an estimate of 100 ng A–D in bee #2. This order is below the LD_{50} of DDT in *A. mellifera* (see above). Although different compounds may display different toxicity in bees, it is rather unlikely that these compounds could produce toxicity in the bees.

Unfortunately, the unknown compounds in the bees could not be traced back to known organohalogen compounds. One option is that they are halogenated natural products (HNPs) which are more and more realized as important contaminants of food and environmental samples (Vetter, 2006). However, HNPs with eight chlorine substituents have not been reported to date.

We propose two possible origins of the unknown and highly chlorinated compounds described in this report. One possibility is that the organochlorines are natural products that the bees collected from unknown sources. The second possibility is that the bees collected other organochlorines that they then used as building blocks for producing the four more highly chlorinated compounds. Of course the role of the four compounds in the life cycle of *E. purpurata* bees, as with all chemicals they collect, is unknown.

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