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Original article

Toxicity of diflubenzuron and penfluron to immature stages of *Apis cerana indica* F and *Apis mellifera* L

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Summary — Diflubenzuron (DF) and penfluron (PF) in acetone were found to be equally toxic to *Apis mellifera* and *A cerana indica* in topical application tests based on equivalent body weights. Toxicity resulting from median lethal dosage was highest for pupae and was lower for IV and III instar larvae. Acetone proved lethal to eggs, I and II instar larvae. There was no delayed lethal and morphological effect of the treatment on larvae, but some adult bees, treated in the same manner as pupae, showed morphological abnormalities, such as crumpled wings and poor interlocking at stylet and lancets of the sting apparatus. Feeding of 50 mg DF to small experimental colonies of both bee species enhanced egg laying but significantly reduced the amount of unsealed and sealed brood within 10 days of treatment.

***Apis mellifera* / *Apis cerana* / diflubenzuron / penfluron / median lethal dose / toxicity / pesticide**

INTRODUCTION

Chitin synthesis inhibitors are gaining popularity in integrated pest management due to their selective action, safety to natural enemies, low mammalian toxicity and lowered tendency to accumulate in food chains (Hammock and Quistad, 1981). These compounds are characterized by contact larvicidal and ovicidal effects on foliage feeders with no systemic activity in plants (Retnakaran *et al*, 1985). Direct and indirect exposure of bees and their brood to extensive usage of one such compound, diflubenzuron, may take place through contact with treated surfaces, contamination of natural food and water sources and utilization of contaminated pollen, honey and water for brood rearing. Low median lethal dose (LD_{50}) of 0.05 μg diflubenzuron (DF) in application tests and 0.12 μg in feeding tests for III instar larvae of *A mellifera* indicate vulnerability of brood (Czoppelt and Rembold, 1981). Wittmann (1981) obtained a 3.5-fold higher toxicity (median lethal dose (LD_{50}): 0.037 μg) in feeding tests. In colonies of *A mellifera* fed with 0.05% (125 ppm DF)

Dimilin, Tomic *et al* (1983) reported reduced mortality (10%) in unsealed compared to sealed brood (68%). Conversely, Gromisz (1981) observed no toxic effects when 0.1% Dimilin (250 ppm DF) was fed to *A mellifera*. Barker and Taber (1977) reported that 59 ppm DF in sugar syrup significantly affected production of sealed brood in this species. Reduced consumption of water and pollen, less brood and fewer new workers and eggs were reported for colonies fed with 100 ppm DF in water (Barker and Waller, 1978). Thus some contradictory information is available on toxicity of DF to *A mellifera*, although the toxicity of this compound or penfluron (PF) to the Indian honeybee, *A cerana indica* F, has not yet been investigated.

MATERIAL AND METHODS

Developmental stage toxicity

The studies were conducted in a mixed apiary consisting of Indian and Italian honey bees at Nauni, Solan, during 1987. Toxicity of DF (2, 6-dichloro-N-[[[4-chlorophenyl)amino]-carbonyl] benzamide and PF(2,6-difluoro-N-[[[4-(trifluoromethyl) pheny] amino]-carbonyl] benzamide) by topical application to both the bee species was obtained by determining medial lethal dose (LD_{50}) following the standard procedure of probit analysis (Finney, 1952). Both compounds were topically applied by an Arnold microapplicator in 1 µl acetone per individual. The temperature at the time of application varied between 28 and 30 °C.

Approximately 70 eggs of 0, 1 and 2 d age of each bee species were treated inside the comb cells with 0.2, 0.5, 1, 2 and 4 µg DF and PF. Two sets of controls were maintained, one with acetone, and the other untreated. Hatching success was recorded at 24-h intervals. Using the criterion of larval age to determine their instar (as detailed by Dogra *et al* (1977) for *A mellifera* and Mishra (1979) for *A c indica*, I through IV instar larvae were treated. Treatments were applied on the dorso-lateral aspect of the body in marked comb cells with 5–6 doses of each chemical in a predetermined dosage range (0.5–10 µg/larva) that resulted in 15–85% mortality in treated lots. With each dose, 40–150 larvae of an instar were treated. Treated larvae subsequently removed by bees (as represented by empty cells) were considered dead. Mortality was recorded after 1, 3 and 5 days of treatment. The portions of sealed comb cells in which treated larvae could have pupated were cut off and fixed in the frame (14 x 12 cm) of experimental cages (18.5 x 10 x 18 cm). These cages were kept in an incubator at 32 ± 1 °C. Mortality at the pupal stage was recorded and any abnormalities in the emerging adults were noted. Five-day mortality data were subjected to probit analysis, since maximum mortality due to chemical treatment occurred within 5 days.

Twenty to 25 freshly pupated workers were treated *in situ* on the head region with each of 4 doses of DF and PF in the range of 1–6 µg chemical/pupa. Treated pupae in comb cells were kept in the experimental cages described above at 32 ± 1 °C in an incubator. Observation on percent mortality in the pupal stage and abnormalities in emerged adult bees were recorded.

Feeding of diflubenzuron in sugar to bee colonies

Two colonies each of *A mellifera* and *A c indica* of 4-frame bee strength were selected. Each colony was provided with one liter of 50% sugar syrup blended with 200 mg Dimilin 25% WP (50 ppm DF) in tin containers with perforated lids. The remaining 4 colonies (2 from each bee species) served as controls and were fed with 1 liter of 50% sugar syrup. For each colony, data on the following parameters were recorded: the number of cells with eggs, larvae, sealed brood, honey and pollen stores on each frame before and 10 d after exposure to DF contaminated sugar syrup. Data were analysed by Student's *t*-test for small samples.

RESULTS AND DISCUSSION

Developmental stage toxicity

All acetone-treated control eggs, and I and II instar larvae were removed by worker

bees within 24 hours of treatment. Acetone proved lethal to the aforementioned groups. Conversely, III and IV instars were not significantly affected by acetone with up to 8% mortality recorded in *A mellifera* and 12% in *A c indica*. A differential dose-dependent mortality response to both chemicals was observed in III and IV instar larvae and pupae.

Treatment of III and IV instar larvae

Most of the treated III instar larvae of *A mellifera* (59 and 80% at the highest dose of 5 µg DF and PF) and *A c indica* (59 and 85% at the highest tested dose of 4 µg DF and PF) died within 1 d of exposure. Presumably, since development in the III instar continues for approximately 1 day in both *A mellifera* (Dogra *et al,* 1977) and *A c indica* (Mishra, 1979), treated larvae at higher doses had died either during or after ecdysis to IV instar, as demonstrated in many insect species (Retnakaran *et al,* 1985). Additional mortality was observed after 3 d of treatment at low doses. In *A mellifera*, DF and PF at 1 µg/larva caused 10 and 11% mortality after 1 d and 25 and 20% after 3 d. For *A c indica*, mortality resulting from the 0.5 µg dose was 16 and 22% in 1 d and 28 and 29% after 3 d. Surviving IV instar larvae may have died by the end of the instar period (duration about 2 d) thereby maximising the 3-d mortality. No mortality was observed during the pupal stage. For III instar larvae of *A mellifera*, the LD_{50} values were 2.42 and 2.58 µg for DF and PF. In comparison, the respective values of 1.49 and 1.56 µg/larva for *A cerana* were quite low (figs 1A,B; table I). For DF, the LD_{50} value was 6.01 µg for IV instar *A mellifera* larvae and 3.65 µg for IV instar *A c indica* larvae. Comparative values for PF were 6.15 and 3.54 µg/IV instar larva (table I; figs 1C,D).

Probit analysis revealed a narrow dosage range (0.5–5 µg for III and 2–10 µg for IV instar larvae) for both compounds, causing 20–90% mortality in both species (figs 1A–1D). Significantly, a lower median lethal dose was recorded in *A c indica* than

Table I. A comparison of median lethal doses (LD_{50}) of diflubenzuron (DF) and penfluron (PF) applied to larvae and pupae of *A mellifera* and *A c indica*.

Treatment stage		*Median lethal dose (LD_{50})*							
		A mellifera				A c indica			
		µg/larva	*95% fiducial limits*	*µg/g body weight*	*b (slope)*	*µg/larva*	*95% fiducial limits*	*µg/g body weight*	*b (slope)*
Third instar larva	DF	2.42	2.03 & 2.87	52.90	2.388	1.49	1.16 & 1.91	56.15	2.072
	PF	2.58	2.15 & 3.10	56.49	2.419	1.56	1.21 & 2.01	58.97	1.890
Fourth instar larval	DF	6.01	5.49 & 6.58	45.51	4.340	3.65	3.25 & 4.09	49.13	3.930
	DF	6.15	5.65 & 6.70	46.57	4.908	3.64	3.18 & 4.16	49.02	3.220
Pupa (freshly formed)	DF	3.01	2.41 & 3.74	22.33	3.435	2.04	1.47 & 2.83	22.69	2.130
	PF	3.09	2.54 & 3.76	22.96	3.874	2.57	1.84 & 3.58	28.58	2.170

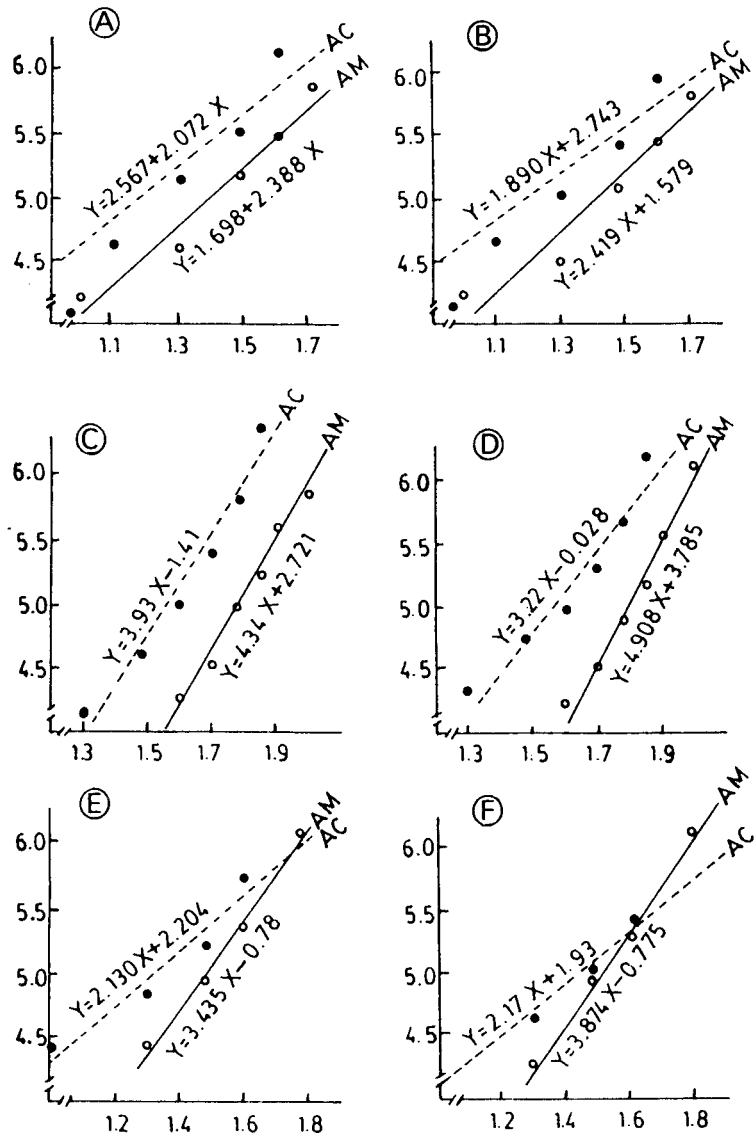

Fig 1. Dosage–mortality response for topically applied diflubenzuron (1A, 1C and 1E) and penfluron (1B, 1D and 1F) to third instar (1A; 1B), fourth instar larvae (1C; 1D) and pupae (1E; 1F) of *Apis cerana* (AC) and *Apis mellifera* (AM). On abscissa is the log (dose μg x 10) value and ordinate refers to probit mortality.

in *A mellifera*. *A mellifera* larvae of the same age (weight 45.67 and 131.99 mg/larva for III and IV instar) are 1.7–1.8-fold heavier than those of *A c indica* (26.52 and 74.22 mg). Larger, heavier animals require a higher dose than smaller animals and

a means of comparison is *via* body weight (Busvine, 1971). LD_{50} expressed as equivalent body weight (µg/g body weight) was marginally higher for *A c indica* larvae than for *A mellifera* larvae (table I). The lower slope of log dose-probit mortality regression lines for *A c indica* and the convergence of these lines for the 2 bee species reveal that the Indian bee is comparatively tolerant to these chemicals. Both compounds exhibit comparable toxicity for each bee species with a distinct overlap in 95% LD_{50} fiducial limits. Conversely, several studies have found PF to be more toxic than DF to some insect species (Chang, 1979; Grannet *et al,* 1983; Thakur and Kumar, 1984). Presumably, the larval cuticle of the honeybee is not an effective barrier to penetration of DF or PF.

Czoppelt and Rembold (1981) obtained very low LD_{50} (50 ng or 0.05 µg) in application tests, compared with 2.42 µg/III instar larva found in the present study. Comparison of LD_{50} for 2 instars of both species on equivalent body weight basis showed IV instar larvae to be more susceptible than III instar. Similar developmental stage sensitivity has also been recorded for larvae of *Choristoneura fumiferana* (Grannet and Retnakaran, 1977), *C occidentalis* (Rappaport and Robertson, 1981), and *Spodoptera frugiperda* (Sagiston and Almedia, 1982). However, earlier instars of *Operophtera brumata* and *Erannis bajara* (Lecheva, 1985) and *Corcyra cephalonica* (Mayuravalli and Reddy, 1986) treated with DF and *Spodoptera litura* treated with another chitin synthesis inhibitor, triflumuron (BAY SIR 8514; Natrajan *et al,* 1988) are reported to be more susceptible than later instars; this lower susceptibility appears to be due to an increase in size of the insect. Under such circumstances it is better to make a comparison on an LD_{50}/unit body weight basis (Busvine, 1971).

Treatment of pupae

Median lethal dose of both compounds per pupa was not significantly higher in *A mellifera* than in *A c indica*; the LD_{50} for DF and PF was also comparable in the 2 species (table I). On an equivalent body weight basis, *A c indica* pupae were less susceptible to PF compared to *A mellifera*. The regression lines for pupae of both species intersect such that the slope of the line for *A c indica* is less than that for *A mellifera* (figs 1E,F). Therefore, *A mellifera* is more sensitive than *A c indica* to increases in dose.

In pupae of *A mellifera* treated with 6 µg DF per pupa adult emergence was 13%, with 9% workers showing morphological defects. Treatment with 4 µg DF produced 33% worker bees, of which 5% had morphological abnormalities. In these bees, a white lump of unrecognisable identity was detected entangled with the stylet and lancets of the sting apparatus (fig 2). This may have been the evaginated reproductive system or shed cuticle from the integument or the hindgut of the pupa. However, apart from the stylet and the lancets which were not properly interlocked, the sting apparatus appeared normal. In pupae of *A c indica* treated with 4 µg DF each, adult

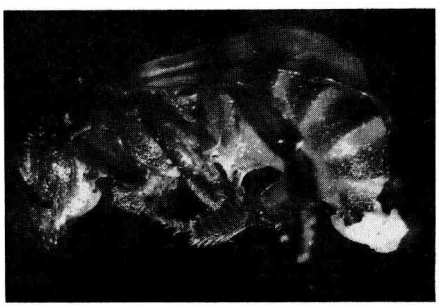

Fig 2. Adult worker emerged from diflubenzuron treated pupa with a lump of white tissue at the abdominal end.

mergence was only 22% and in half of tthe group a similar lump of tissue was observed with the sting apparatus. *A mellifera* adults that emerged from 6% of the pupae treated with 6 μg PF had crumpled wings, but no such deformity was noticed in the emerged adults of the Indian bee.

Feeding of diflubenzuron in sugar syrup to small bee colonies

In both the control and treated colonies of each bee species the quantity of eggs, unsealed and sealed brood and honey and pollen stores did not differ significantly in the pretreatment count (table II). However, after 10 d DF treatment, there was a significant increase in the number of cells containing eggs in both species in treated colonies as compared with the pretreatment count. In control colonies there was a decrease in number of eggs. Lineva and Chunina (1980) also recorded more egg laying by females of *Musca domestica* fed on DF, but all laid eggs were non-viable. In treated bee colonies an increased egg count may be a consequence of additional space available for the queen due to larval death. The increased egg count is not due to an accumulation of non-viable eggs in combs, as bees removed such eggs. Barker and Waller (1978) also obtained more

Table II. Effect of feeding of diflubenzuron (50 ppm) on development of *Apis mellifera* and *A cerana indica* colonies.

Parameters		*A mellifera*			*A c indica*		
		Average No of cells/frame (N = 8)		*% increase (+) or decrease (–) over pretreatment count*	*Average No of cells/frame (N = 8)*		*% increase (+) or decrease (–)*
		Before treatment	*After treatment*		*Before treatment*	*After treatment*	
Eggs	T	310.4	425.5*	+ 37.1	220.4	371.6*	+ 65.6
	C	282.3	216.0	– 23.1	367.8	240.4	– 34.6
Unsealed brood	T	254.8	52.0*	– 79.6	194.1	31.8*	– 83.6
	C	197.9	271.9	+ 37.4	363.8	349.6	– 3.9
Sealed brood	T	521.0	93.6*	– 82.0	433.9	69.6*	– 83.9
	C	270.1	257.1	– 6.9	507.4	493.1	– 2.8
Total brood	T	77.5.8	145.6*	– 81.2	628.0	47.6*	– 92.4
	C	474.0	507.8	+ 7.1	880.1	833.8	– 5.3
Honey	T	271.4	108.8	– 55.9	307.8	203.0	– 34.0
	C	166.4	76.9	– 53.8	705.0	420.0	– 40.4
Pollen	T	77.5	81.9	+ 5.6	139.4	120.6	– 13.5
	C	55.4	51.8	– 6.5	114.8	73.8	– 35.7

* Treatments differ significantly from control in Student's *t*-test at $P = 0.05$. T = treated colony; C = control/untreated colony.

eggs in the combs of *A mellifera* colonies fed on sugar syrup containing 1 000 ppm DF.

Treated colonies of *A mellifera* and *A c indica* had significantly reduced unsealed (79.6 and 83.6%), sealed (82 and 83.9%) and total (81.2% and 93.4%) brood in post-treatment observation (table II). However, in control colonies, there was a slight increase (7.1%) in the brood of Italian and a marginal decrease (5.3%) in the brood of Indian bee. The larvae in unsealed cells are fed by nurse bees and contaminated food is likely to prove fatal. Barker and Waller (1978) reported less brood and fewer new workers in an *A mellifera* colony fed on 100 ppm DF in water. Conversely, Tomic *et al* (1983) obtained less (10.2%) reduction in the unsealed and a high reduction (68%) in the sealed brood in small experimental colonies of the Italian bee fed on 125 ppm DF. However, Gromisz (1981) failed to observe any toxic effect when 0.1% Dimilin (250 ppm DF) was fed to an *A mellifera* colony. In the present study, 50 ppm DF proved more toxic to *A c indica*, compared to *A mellifera*, because the former is smaller in size.

Treatment had a non-significant effect on honey store of the colony. A reduction in honey store in treated and control colonies of the two bee species was due to inclement weather conditions at the time of the experiment. In the pollen store a marginal increase (5.6%) was found in treated colonies of the Italian bee compared to a slight decrease (6.5%) in the control groups. In the case of the Indian bee, the pollen store was reduced by 13.5 and 35.7% in the treated and control colonies, respectively. Reduction in the pollen stores was apparently due to consumption of pollen for brood rearing. Barker and Waller (1978) also reported less consumption of water and pollen and less brood in the colony fed on 100 ppm DF in water.

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Résumé — Toxicité du diflubenzuron et du penfluron vis-à-vis des stades immatures d'*Apis cerana indica* F et d'*Apis mellifera* L. Des larves de 3e et 4e stades de l'abeille indienne *Apis cerana indica (A c)*, et de l'abeille italienne, *Apis mellifera (A m)*, ont reçu des applications topiques d'une solution acétonique de diflubenzuron (DF) et de penfluron (PF), inhibiteurs de la synthèse de la chitine. Pour des doses semblables les larves d'*A c* sont plus sensibles (DI_{50} en µg/abeille) que celles d'*A m* (fig 1A-D). Pourtant, si l'on se base sur l'équivalent poids corporel (DI_{50} en µg/poids corporel) les larves des 2 espèces ont plus ou moins la même sensibilité, la DI_{50} de l'abeille indienne étant légèrement plus élevée (tableau I). Les nymphes des 2 espèces présentent la même sensibilité (fig 1E-F) mais, sur la base de l'équivalent poids corporel, celles de l'abeille indienne sont un peu moins sensibles que celles de l'abeille italienne (tableau I). Chez les 2 espèces les stades peuvent être classés par ordre décroissant de sensibilité à ces produits : nymphes, larves du 3e stade et larves du 4e stade. DF et PF ont donc la même toxicité pour les 2 espèces. La toxicité vis-à-vis des œufs et des larves du 1er et du 2e stade n'a pas pu être déterminée à cause de l'action mortelle de l'acétone. Les traitements n'ont eu aucune action létale ou morphologique différée chez les larves, la plupart des individus mourant dans les 3 j suivant le traitement. L'application de 6 et de 4 µg de DF sur les nymphes a provoqué chez plus de la moitié des adultes des 2 espèces des modifications morphologiques. Les abeilles présentaient des boules de tissu blanc d'origine

inconnue à l'extrémité de l'abdomen, qui empêchaient le fonctionnement normal du dard. Les nymphes d'abeilles italiennes traitées avec 6 µg de DF ont donné naissance à des abeilles qui, dans 6% des cas, possédaient des ailes rabougries. De petites populations d'abeilles (4 cadres) des 2 espèces ayant reçu 50 mg de DF (200 mg de Dimilin dans 1 l de sirop de sucre à 50%) ont eu, dans les 10 j suivants, une ponte accrue et une réduction significative du couvain, operculé ou non; par contre le traitement n'a pas influé en général sur les provisions de miel et de pollen (tableau II).

***Apis cerana* / *Apis mellifera* / pesticide / toxicité / dose létale 50**

Zusammenfassung — Die toxische Wirkung von Diflubenzuron und Penfluron gegenüber unreifen Stadien von *Apis cerana indica* F und *Apis mellifera* L. Bei lokalen Anwendungsversuchen mit Lösungen der Chitinhemmer Diflubenzuron (DF) und Penfluron (PF) in Azeton waren Larven des 3 und 4 Häutungstadiums der Indischen Honigbiene *Apis cerana indica* für gleiche Dosen empfindlicher (LD_{50} in µg/Biene; Abbildungen 1–4) als Italienische Bienen von *A mellifera*. Auf der Basis des äquivalenten Körpergewichtes (LD_{50} in µg/g Körpergewicht) waren jedoch die Larven beider Arten mehr oder weniger gleich empfindlich, wobei sich die LD_{50} der Indischen Biene als geringfügig höher erwies (Tabelle II). Die Puppen beider Arten waren gleich empfindlich (Abbildungen 5–6), aber auf der Basis des Körpergewichts waren die Puppen der Indischen Bienen weniger empfindlich als die der Italienischen Biene (Tabelle I). Die Empfindlichkeit beider Bienenarten in fallender Reihenfolge für diese Präparate war Puppen, Larven des dritten und Larven des vierten Stadiums. DF und PF hatten im Wesen dieselbe toxische Wirkung auf beide Bienenarten. Die toxische Wirkung auf Eier und Larven des 1 und 2 Häutungsstadiums konnten wegen der tötlichen Wirkung des Lösungsmittels Azeton nicht bestimmt werden. Es gab keine verzögerten letalen oder morphologischen Wirkungen bei Behandlungen der Larven, die meisten Individuen starben innerhalb von drei Tagen nach der Behandlung. Die Anwendung von 6 und 4 µg DF auf Puppen der Italienischen und der Indischen Biene führten bei mehr als der Hälfte der geschlüpften Tiere zu morphologischen Veränderungen. Diese Bienen zeigten einen weißen Gewebeklumpen der die Funktion von Stylett und Lanzetten des Stachelapparates behinderte. Eine andere Mißbildung waren verkrüppelte Flügel, die nach Behandlung von Puppen der Italienischen Biene mit 6 µg DF bei 6% der geschlüpfen Arbeiterinnen beobachtet werden konnten.

Nach Fütterung kleiner Versuchsvölker von 4 Waben beider Bienenarten mit 50 mg DF (200 mg Dimilin in einem Liter Zuckersirup 50%) führte innerhalb von 10 Tagen zu gesteigerter Eiablage und einer signifikanten Verminderung der offenen und verdeckelten Brut, aber im allgemeinen zu keiner Veränderung der Honig- und Pollenvorräte (Tabelle II).

Aus diesen Studien geht deutlich hervor, daß es beim Vergleich der toxischen Wirkung einer chemischen Verbindung auf verschiedene Entwicklungsstadien besser ist, die Ergebnisse auf der Basis des Körpergewichtes auszudrücken, und daß Penfluron für beide Arten gleich toxisch ist wie Diflubenzuron. Obwohl die Wahrscheinlichkeit, daß Trachtbienen 50 mg Diflubenzuron in den Stock eintragen gering ist, könnte sich die Verunreinigung von Nahrung und Wasser für die Larven als schädlich erweisen.

***Apis mellifera* / *Apis cerana* / Pestizide / toxische Wirkung / letale Dose 50**

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