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Original article

Effects of diflubenzuron and penfluron on workers of *Apis cerana indica* F and *Apis mellifera* L

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Summary — Newly emerged adult workers of *Apis mellifera* and *A cerana indica* tolerated a topically applied dose of 100 µg diflubenzuron (DF) and penfluron (PF) in acetone but the treated bees weighed less than control bees at 2 and 6 d of age. Oral administration of 100 µg DF (as Dimilin 25% wettable powder) in 10 µl sugar syrup proved fatal to *A c indica*. After 6 d of feeding 50 µg DF, hypopharyngeal gland development, measured as size of acini, was significantly suppressed in both bee species. The suppressed gland development in the treated group could be a consequence of poor gain in weight. Foragers of both bee species readily accepted DF-contaminated sugar syrup and, with increasing doses, there was decrease in time required to consume the contaminated sugar syrup in a dose-dependent manner. The treated bees weighed significantly less than the control bees. Thus, at higher doses chitin synthesis inhibitors may also prove harmful to adult bees.

***Apis mellifera* / *Apis cerana indica* / toxicity / insecticide / diflubenzuron / penfluron**

INTRODUCTION

Diflubenzuron is recognized as an ecologically safe and specific-action insecticide owing to its chitin synthesis inhibition properties. It is relatively safe to honey bees and when it was applied topically to the workers of the European honeybee, *Apis mellifera* L, a median lethal dose higher than 30 µg/bee was observed (Stevenson, 1978). Usha and Kandasamy (1986) categorized diflubenzuron (DF) as the safest insecticide to the adult Indian bee, *A cerana indica* Fabr and, following exposure of adult bees for 90 min to surfaces treated with as much as 10 000 ppm (10 g l^{-1}) DF, there was no mortality. Except for this published work, no information is available on the effect of DF and another potent chitin synthesis inhibitor, penfluron (PF), on the 2 bee species. Short-term experiments based on mortality data may not give true picture of safety of a chemical to bees and, at sublethal doses, the treatment may have latent effect as demonstrated by El-Din *et al* (1990). The toxicity of DF and PF to developing stages of *A c indica* and *A mellifera* has been reported by Chandel and Gupta (1992). This

article reports the work conducted with young and foraging workers of the two bee species.

MATERIALS AND METHODS

Treatment of newly emerged bees

Newly emerged adult bees, which were lethargic, dull in colour and had unprotrudable stings, were removed from the hive, numbered on the thorax by adhering a circular paper disk (4 mm diameter) bearing a number, and individually weighed on the electronic balance. Twenty-five to 30 bees of each species were treated with 100 μg DF or PF in acetone on their abdomen. Half of the treated bees of each species were consigned to a cage (13.5 x 11 x 13 cm) with a hole at the top through which gravity feeder containing 50% sugar syrup was inserted half an hour after the treatment. Thus, each treatment was represented by 2 cages. All the test cages were maintained in an incubator at 29 ± 1°C. Observations on the gain in weight were recorded 2 and 6 d after the treatment.

Experiments with oral feeding of the chemical were restricted to DF (used as Dimilin, 25% wettable powder), since formulated material of PF was not available. *Per os* administration of 100 and 50 μg DF was made 1 h after the capture to each of the 30 newly fledged bees of both species through 10 μl of sugar syrup with the help of calibrated capillary tubes. These were handled as described above and maintained for 6 d, until the acini of hypopharyngeal gland were well developed in the normal bees taken from the hive. Ten bees of each treatment set were made immobile by cold treatment in the refrigerator for 15 min and dissected in physiological saline containing 0.145 M NaCl, 0.006 M KCl, 0.002 M $CaCl_2$ and 0.002 M $NaHCO_3$ to expose the hypopharyngeal glands *in situ*. The cut-open head of the bee was charged with 10% V/V formal saline (saline with 10% formaldehyde) for 1 h, the glands were removed and washed thoroughly with water to eliminate the formal saline. These were stained in borax carmine for 15–20 min, washed in water, dehydrated through ascending series of alcohol (15 min in each grade), cleared in clove oil and xylene, and mounted in DPX® (Glaxo l aboratories India Ltd, Bombay, India) on glass slides. Gland development was determined by measuring the length and diameter of 10 acini of each gland at random under a microscope by an ocular lens precalibrated with a standard micrometer. Each mean value of a treatment represented 200 measurements (10 acini/gland, 2 glands in a bee, and 10 bees as replicates). Six-day-old hive workers (10 in number) were also handled in the same way to compare their gland development with the laboratory-maintained 6-day-old bees.

Oral administration of diflubenzuron to foragers

Outgoing foragers of both the bee-species were captured at the hive entrance in an insect collection net and, after an hour, each of the 30 bees was fed 10 μl of 50% sugar syrup containing a known amount of DF through calibrated capillary tubes. The time taken by a worker bee ($N = 15$) to consume the provided sugar syrup was recorded with the help of a chronometer. Each cage was fitted with a gravity feeder containing a known volume of sugar syrup. Test cages were maintained for 4 d and the weight gain of experimental bees was recorded. Data were subject to *F*- and *t*-tests and treatment responses were compared by using the least significant difference concept (LSD) as outlined by Gomez and Gomez (1984).

RESULTS

Weight gain in topically treated bees

Newly emerged workers of *A mellifera* (average weight 82.03 mg ± 1.96 SE) were approximately 1.5 times heavier than *A c indica* (56.23 mg ± 1.37 SE) but both bee species tolerated doses of DF and PF as high as 100 μg per imago. Within 2 d, the effect of the treatment at this dose was evident (table I) and the treated bees weighed significantly less than the controls.The weight gain of the treated bees was significantly lower in 6-day-old bees (119.6 and 121.7% of the initial weight of *A mellifera* and *A c indica* in the control against 110.5

Table I. Weight gain of newly emerged bees treated with 100 μg diflubenzuron and penfluron.

Bee species and treatment	*Average weight of bees in mg*		
	day 0	*day 2**	*day 6**
A mellifera (*N* = 23)			
Control	83.9	90.90[a]	100.37[a]
DF	81.08	85.83[b]	89.63[c]
PF	81.11	84.73[b]	94.77[b]
LSD (*P* = 0.05)	NS	4.084	5.598
A c indica (*N* = 25)			
Control	53.58	65.10[a]	70.03[a]
DF	58.67	60.82[b]	63.55[b]
PF	56.44	61.36[b]	66.31[b]
LSD (*P* = 0.05)	NS	2.84	3.27

NS = non-significantly different; * figures followed by the same letter at each day of observation for a bee species do not differ significantly.

and 108.3% with DF and 116.8 and 117.5% with PF treatment). In the control and treated lots, the percentage mortality recorded 6 d after the treatment did not vary significantly but higher mortality was registered with DF (23.3 and 16.7% for *A mellifera* and *A c indica*) than with PF (0 and 6.7%) or control (16.7 and 7.1%).

Size of acini of hypopharyngeal glands in nurse bees fed diflubenzuron

In the laboratory control, the diameter and length of acini were 22.1 and 11.8% smaller in *A mellifera* and 33.4 and 34.7% smaller in *A c indica* than in those taken from the hive

Table II. Effect of oral administration of diflubenzuron to freshly emerged bees on the hypopharyngeal gland development at 6 d of age.

Dosage (μg/bee)	*Hypopharyngeal gland development in terms of size of acini (μm) **			
	Length		*Diameter*	
	A mellifera	A c indica	A mellifera	A c indica
Control (hive)	174.0[a]	159.5[b]	106.8[x]	100.8[x]
Control (laboratory)	153.4[b]	104.2[c]	83.2[y]	67.1[yz]
50	101.6[c]	86.8[d]	58.2[z]	51.2[z]
100	100.8[c]	**	57.4[z]	**
LSD (*P* = 0.05)	13.2		15.9	

* Figures with the same letters do not differ significantly; ** complete mortality occurred.

(table II). Acini of nurse bees from the colony had opaque and pale appearance but these were translucent and whitish in the laboratory-maintained control. Thus, even the control bees under hive conditions and in cages showed significant differences in the sizes of acini. Although bees in the laboratory control were also provided with the pollen from combs, feeding was negligible.

In *A mellifera* taken from the hive and fed with 100 and 50 μg DF per bee, the acini were significantly smaller than in the laboratory control (table II). The acini of *A c indica* nurse bees fed with 50 μg DF were even smaller. However, feeding of 100 μg DF/*A c indica* bee proved fatal.

Feeding diflubenzuron to foragers through sugar syrup

Both bee species readily accepted the DF-contaminated sugar syrup. With an increase in dose from 25 to 100 μg per 10 μl sugar syrup, there was a decrease in the time taken to consume the contaminated syrup from 9.18 to 6.97 s in *A mellifera* and 9.67 to 6.83 seconds in *A c indica*, as compared with 13.7 s (r = −0.988) and 13.16 s (r = −0.999) in their respective controls (table III).

The foragers of both bee species fed with DF weighed significantly lower than the control bees (table IV). *A mellifera* tolerated a dose as high as 100 μg but most of *A c indica* bees died at 100 μg. The weight in *A mellifera* was significantly lower at 12.5 and 25 μg doses (4.9 and 11.1% less than the control). However, at 100 μg DF, there was a minimal weight of the forager (21.4% reduction over control) and the weight at 50 μg dose (18.4% reduction) did not differ significantly from that at 100 μg. For *A c indica*, all treatments differed significantly from one another and reduction in weight over the control bees was slightly higher compared with *A mellifera*, being 6, 14.6 and 20.3% at 12.5, 25 and 50 μg dose/bee, respectively.

DISCUSSION

Benzoyalphenyl urea compounds as chitin synthesis inhibitors are relatively safe to non-target beneficial arthropods (including

Table III. Time taken by foragers to consume diflubenzuron contaminated sugar syrup.

Amount fed (μg) to each bee (N = 15)	*Time taken (s) to consume 10 μl sugar syrup**	
	A mellifera	A c indica
Control	13.70[a]	13.16[a]
25	9.18[b]	9.67[b]
50	8.37[b]	7.98[c]
100	6.97[c]	6.83[c]
LSD (P = 0.05)	1.89	1.44

* Figures in a column with the same letter do not differ significantly from each other.

Table IV. Effect of oral administration of diflubenzuron on weight on foragers after 4 d of ingestion.

Dosage (μg/bee)	*Average weight (mg)**	
	A mellifera	A c indica
Control	105.66[a]	74.46[a]
12.5	100.45[b]	70.00[b]
25	93.97[c]	63.58[c]
50	86.19[d]	59.38[d]
100	83.09[d]	–
LSD (P = 0.05)	4.65	3.36

* Figures in a column followed by the same letter do not differ significantly from each other.

honeybees) and, where harm occurs, the routes of chemical uptake presumably are through water or nectar feeding and possibly some cuticular absorption (Retnakaran *et al*, 1985). DF and PF are both toxic to the immature stages of *A mellifera* and *A c indica* and feeding of DF to experimental colonies reduces the brood (Chandel and Gupta, 1992). However, delayed effect of these compounds on young bees and foragers were evident at high doses and appeared as a poor gain in weight of treated bees and smaller hypopharyngeal glands in young workers.

Bees readily accepted the DF-contaminated sugar syrup. Faster ingestion of DF contaminated sugar syrup could either be due to increased palatability and acceptance owing to addition of the formulated product or stimulation of the feeding centres. The subsequent feeding of sugar syrup during the 4 days of the experiment was not affected. The weight gain of newly emerged bees topically treated with DF and PF and of foragers fed with DF was comparatively low. Even oral feeding proved more deleterious than local application of the DF as *A c indica* could tolerate 100 µg dose topically but not by the oral route. As observed in the present case, El-Din *et al* (1990) also found latent effects of DF and other chitin synthesis inhibitors, such as reduced body weight of adults (19.3–39.6%) emerged from treated larvae of *A m carnica*. The poor weight gain of treated bees could be the effect of the treatment on peritrophic membrane which is well formed predominantly in the anterior part of the midgut and is supplemented by delamination further back (Richards and Davies, 1977). Treatment is known to slow down peritrophic membrane production in Orthoptera (Clarke *et al*, 1977; Becker, 1978). The other effect of the treatment could be on the enteric epithelium (Ker, 1978), the depression in activity of gut enzymes like invertase (Ishaaya and Ascher, 1977), or impaired digestion and assimilation as reported for lepidopterous larvae (Radwan *et al*, 1986; Reed and Bass, 1979; Velpandi *et al*, 1986). A faster loss in weight of pupae of *Corcyra cephalonica* treated with DF (Sriramulu and Mehrotra, 1987) and adult *Tenebrio molitor* (Soltani *et al*, 1984) has also been observed.

In young bees, another effect observed was less development of hypopharyngeal glands in the treated bees at 6 d of age when these glands were fully developed in the normal bees. According to Cruz-Landim and Silva de Moraes (1977), secretion in these glands starts about 6 d after emergence and continues for about 12 d. The size of acini (diameter x length) of hypopharyngeal glands, taken as a criterion to measure their development and nursing activity, reveals that the acini of bees from laboratory control were smaller than those of the bees in the hive. In the colony, bees initiate pollen consumption about 2 h after emergence (Dietz, 1969); 2-d-old bees are fed more by older bees than by themselves and maximum consumption is at 5 d of age (Morton, 1950; Pain, 1961). The pollen consumption is apt to enhance the general body protein-metabolism and its activity in the hypopharyngeal gland. Gland development was suppressed in treated bees as compared with laboratory control bees. The suppressed gland activity in the treated lot could be a consequence of poor gain in weight. A decrease in body protein of II instar larva of *Zaprionus paravittiger* (Chopra and Rup, 1985) and protein of peritrophic membrane in *Locusta migratoria* (Clarke *et al*, 1977), and 9–53% loss of protein in *Heliothis armigera* and *H zea* larvae fed on DF (Elherrawie *et al*, 1985) have also been reported. However, Barker and Taber (1977) found no evident abnormality in hypopharyngeal glands of adult bees fed DF at 59 ppm in sugar syrup, which is contrary to our observations.

The present study indicates the possible latent effect of chitin synthesis inhibitors on adult honeybees. Herbert *et al* (1986) suggested feeding of another chitin inhibitor, Baysir 8514, through pollen substitute for disrupting brood production for 2–3 weeks to eliminate ectoparasitic mites reproducing on the brood. However, such a treatment may have unremarked slow deleterious effect on colonies and before making any such recommendation, detailed investigations need to be carried out on the sublethal effects of chitin synthesis inhibitor in honeybee colonies.

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Résumé — Action du diflubenzuron et du penfluron sur les ouvrières d'*Apis cerana indica* F et d'*Apis mellifera* L. Des ouvrières naissantes (*N* = 25-30) d'*Apis mellifera* et d'*Apis cerana indica* ont été traitées individuellement par voie topique avec 100 µg de diflubenzuron (DF) ou de penfluron (PF) dilués dans 25 µl d'acétone, maintenues dans des cagettes à 29 ± 1°C et nourries avec du sirop à 50 % et du pollen prélevé dans les rayons. Les abeilles ont supporté le traitement mais 2 j plus tard les premiers effets sont apparus : le poids des abeilles traitées était significativement inférieur à celui des témoins (tableau I). À l'âge de 6 j le poids était encore plus bas, en particulier après traitement au DF. Le diflubenzuron (sous forme de dimilin à 25% en pourcentage de poids) a aussi été administré par voie orale à de jeunes ouvrières (*N* = 30) récemment maintenues en étuve à 29 ± 1°C durant 6 j : 10 µl de sirop contenant 50 ou 100 µg de DF étaient présentés aux abeilles dans des tubes capillaires calibrés. L'activité des glandes hypopharyngiennes a été évaluée d'après la taille (diamètre x longueur) des acini. Les glandes hypopharyngiennes de 10 abeilles par lot traité et par lot témoin ont été prélevées et fixées pendant 1 h dans une solution aqueuse à 10% de formaldéhyde, colorées pendant 15-20 min au carmin boracique, déshydratées à l'alcool, nettoyées à l'essence de girofle et au xylène, puis montées dans du DPX®. Chaque moyenne représente 200 mesures (10 acini/glande, 2 glandes/abeille sur 10 abeilles). On a traité de la même façon des ouvrières âgées de 6 j (*N* = 10) provenant de la ruche pour comparer l'activité de leurs glandes avec celles des abeilles de même âge maintenues au laboratoire. Dans le lot témoin du laboratoire les acini étaient plus petits que ceux des abeilles de la ruche (tableau II). Pourtant les abeilles des 2 espèces ayant ingéré 50 µg de DF avaient des acini significativement plus petits que ceux des groupes témoins du laboratoire. La réduction de l'activité glandulaire chez les abeilles traitées pourrait être une conséquence de la faible prise de poids due à une perturbation du métabolisme. Les butineuses des 2 espèces ont consommé sans difficulté le sirop additionné de diflubenzuron. Lorsqu'on a augmenté la dose de 25 à 100 µg/100 µl de sirop, le temps nécessaire à la consommation a diminué de 9,18 à 6,97 s pour *A mellifera* et de 9,67 à 6,83 s pour *A c indica*, tandis que pour les témoins il était respectivement de 13,7 et 13,16 s (tableau III). Lorsque les butineuses ont reçu de 12,5 à 100 µg de DF / 10 µl de sirop à 50%, on a observé après 4 j de traitement une perte de poids significative fonction de la dose (tableau IV).

***Apis mellifera* / *Apis cerana indica* / insecticide / toxicité / diflubenzuron / penfluron**

Zusammenfassung — Wirkung von Diflubenzuron und Penfluron auf Arbeiterinnen von *Apis cerana indica* F und *Apis mellifera* L. Frischgeschlüpfte Arbeiterinnen (N = 25–30) von *A mellifera* und *A c indica* wurden einzeln mit 100 µg Diflubenzuron (DF) bzw Penfluron (PF) beträufelt, die jeweils in 25 µl Aceton gelöst waren. Die Arbeiterinnen wurden bei 29°C in Käfigen gehalten und mit 50% Zuckerlösung und Pollen aus der Wabe gefüttert. Die Bienen tolerierten diese Behandlung, aber 2 Tage nach der Behandlung zeigten sich die ersten Effekte: das Gewicht der Bienen war signifikant niedriger als bei den Kontrollen (Tabelle I). Im Alter von 6 Tagen waren die Bienen immer noch leichter, besonders nach der Behandlung mit DF. DF wurde auch oral an 30 frischgeschlüpfte Arbeiterinnen verfüttert, indem 10 µl Zuckerlösung mit 100 oder 50 µg DF in einer calibrierten Kapillare geboten wurde. Die Bienen wurden 6 Tage in kleinen Käfigen bei 29 ± 1°C im Brutschrank gehalten. Die Aktivität der Hypopharynxdrüse wurde durch die Messung der Größe der Acini (Durchmesser x Länge) bestimmt. Dazu wurden Dauerpräparate der Drüsen von 10 behandelten und 10 Kontrollbienen angefertigt. Sie wurden in 10% wässeriger Formaldehydlösung eine Stunde fixiert, 15–20 min mit Borax Carmin gefärbt, in der Alkolholreihe dehydriert, mit Nelkenöl aufgehellt und in DPX® eingebettet. Jeder Mittelwert repräsentiert 200 Messungen (10 Acini/Drüse, 2 Drüsen/Biene von 10 Bienen). Als zusätzliche Kontrolle wurden 10 Bienen im Alter von 6 Tagen aus dem Stock untersucht. Die Kontrollgruppe aus dem Labor hatte kleinere Drüsen als die Stockbienen (Tabelle II). Bei beiden Bienenarten hatten die Arbeiterinnen jedoch signifiant kleinere Acini als beide Kontrollgruppen, wenn sie 50 µg DF erhalten hatten. Die verminderte Drüsenaktivität könnte durch die geringe Gewichtszunahme durch einen beeinträchtigten Stoffwechsel bedingt sein. Sammlerinnen von beiden Bienenarten nahmen bereitwillig Zuckerwasser mit DF (als Dimilin 25% WP Gewichtsprozent) auf. Bei einer Erhöhung der Dosis von 25 auf 100 µg/10µl Zuckerwasser nahm die Zeit der Futteraufnahme von 9,18 auf 6,97 s bei *A mellifera* und von 9,67 auf 6,83 s in *A c indica* ab. Bei der Kontrollgruppe betrug die Zeit der Futteraufnahme 13,6 bzw 13,16 s (Tabelle III). Wurden Sammlerinnen mit 12,5 – 100 µg DF/10 µl 50% Zuckersirup gefüttert, sank ihr Gewicht innerhalb von 4 Tagen signifikant und zwar dosisabhängig (Tabelle IV). Diese Versuche zeigen, daß eine topikale Applikation von DF und PF in höheren Dosen frischgeschlüpfte Arbeiterinnen schädigte. Bei einer Fütterung von DF in Zuckersirup wurde die Aktivität der Hypopharynxdrüse bei frischgeschlüpften Arbeiterinnen vermindert, bei Sammlerinnen erfolgte eine dosisabhängige Verringerung des Gewichts.

Diflubenzuron / Penfluron / Schädigung/ *Apis mellifera* / *Apis cerana indica*

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