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Original article

Effects of four protease inhibitors on the survival of worker bumblebees, *Bombus terrestris* L.

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Abstract – To assess risks posed by transgenic pest-resistant plants, a bumblebee bioassay system was developed. Small and large adults of *Bombus terrestris* were held individually or in groups of 5, 10 or 20 in cages and survival and rates of pollen, sugar and water consumption determined. Effects on bee survival of Kunitz soybean trypsin inhibitor (SBTI), bovine pancreatic trypsin inhibitor (BPTI) and two potato protease inhibitors, POT-1 and POT-2, were determined. SBTI (10 mg·g^{-1}) and POT-1 (10 and 5 mg·g^{-1}) reduced survival significantly. Bees fed POT-2 (10 mg·g^{-1}) had poorer survival than those fed 0.1 or 0.01 mg·g^{-1} POT-2. BPTI had no effect. Untreated bee midguts had elastase-like (283.0 ± 16.9 nmol·min^{-1}·g^{-1} gut), chymotrypsin (148.5 ± 8.4), trypsin (27.2 ± 2.8) and leucine aminopeptidase (258.6 ± 9.6) activities. Elastase-like and chymotrypsin activities were inhibited by SBTI, POT-1 and POT-2, but not BPTI. Trypsin activity was reduced by each inhibitor. Leucine aminopeptidase activity was unaffected.

bumblebee / protease inhibitor / food consumption / small-cage bioassay / pest-resistant transgenic plant

1. INTRODUCTION

Bumblebees are economically significant pollinators of a number of crops grown both outdoors and under glass [25] and they are widely considered as beneficial insects in many natural and agro-ecosystems. With the recent development of a range of transgenic pest-resistant crop plants, there is concern about both the role of insects in the movement of pollen from these plants [8, 9, 21] and about the direct impact of these plants on pollinator health and survival. While there has been a number of studies

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involving honeybees [1, 4, 16, 17, 22, 23] the potential impacts on bumblebees of ingestion of gene products conferring resistance to plant pests have not yet been investigated.

Methods used with honeybees to test the effects of potentially hazardous factors, such as new gene products, are not directly applicable to bumblebees because of a number of biological differences. Because of their larger average body size, each bumblebee is likely to consume a greater amount of any product than a honeybee. Bumblebees do not appear to display the strict temporal polyethism that adult honeybees do, whereby all adults begin as nurse bees and then progress to foraging behaviour. In contrast, there is some evidence that body size is important in determining a bumblebee's role in the colony, i.e. large bumblebees have a greater tendency to forage than small bumblebees [2, 11, 13]. Furthermore, because of species differences in social structure, bumblebees may survive better with fewer companions than honeybees require when held in an incubator in cages. All of these factors will affect bumblebee bioassay design.

The present study had two aims. The first was to develop a laboratory bioassay system appropriate for testing the effects of gene products on adult bumblebees and the second was to use this system to examine the effects on bumblebees of four protease inhibitors (PIs) being incorporated into transgenic plants as pest-resistance factors.

As bumblebee workers vary considerably in body size, a comparison of large and small worker bees was included in the study. The following criteria were considered to be important for the bioassay design: minimum use of the gene product (these may be expensive or difficult to obtain), maximum degrees of freedom for the number of insects used, and maximum bee survival. In the first experiment, small and large bumblebees, *Bombus terrestris*, were kept individually or in groups of 5, 10 or 20 in cages in an incubator. Their survival and the rates at which they consumed pollen, sugar syrup and water were compared. In the second experiment, the effects of feeding bovine pancreatic trypsin inhibitor (BPTI; also known as aprotinin), Kunitz soybean trypsin inhibitor (SBTI) and two protease inhibitors from potato, POT-1 and POT-2, at five different dosage levels, on the survival of caged bumblebees were determined. Furthermore, in order to relate the results of the feeding trials to the effects on the digestive enzymes of the bees, in vitro protease assays of control bumblebee guts were conducted and the effects of the four inhibitors on these proteases determined.

2. MATERIALS AND METHODS

Colonies of bumblebees, each containing approximately 40 adult bees ranging in age from newly-emerged to eight weeks old, were obtained from a commercial supplier (Zonda Resources Ltd, Hastings, New Zealand). These bees had been reared from field-collected queen bees and thus were genetically identical to wild *B. terrestris*. Although the lack of uniformity of adult bee age was expected to be a source of variability in longevity experiments, the colonies were the same type as those used for pollination by greenhouse tomato growers and thus the results may have relevance to those using bumblebees in crop production.

For the first experiment, worker bees were taken from the colonies and assigned to two size classes, designated as "small" (total body length < 1.25 cm) and "large" (total body length > 1.50 cm). Intermediate-sized bees were not used in the experiment. Bees from each size class were randomly assigned to cages either individually or in groups of 5, 10 or 20 bees. The entire experiment was set up on a single day: two bee sizes (small or large) × four groupings (1, 5, 10 or 20 bees) × three blocks (24 cages in total).

The bees were kept in the cages in an incubator at 27 °C, 70% relative humidity,

until all bees were dead. Cages were wooden with two mesh sides and measured 9 × 8 × 6 cm (internal dimensions). Sugar syrup (50% w:v) and water were supplied to the bees via 6 mL graduated gravity feeders. Pollen food (0.33 parts mixed floral pollen, 0.08 parts sodium caseinate, 0.16 parts brewer's yeast, 0.43 parts sucrose, mixed to a paste with water) was supplied in 2 g lots in small open containers. All cages were inspected every two or three days. At each inspection, bee deaths were recorded, the volumes of syrup and water left in the feeders recorded and the pollen food containers weighed. The quantities of syrup, water and pollen consumed were calculated by subtracting the volumes or weights from the previous measurement and dividing by the number of days elapsed and the number of bees alive in the cage at the end of the period. The gravity feeders were replenished, the pollen food containers replaced with fresh ones and any wax cells constructed by the bees were removed.

For the second experiment, bees were assigned to size classes as before, and five large and five small bees were placed in each cage for feeding with PIs. Four different inhibitors, BPTI (from Intergen), SBTI (from Sigma), POT-1 and POT-2 (extracted from potatoes according to [3, 20]) were used at five different dosage levels each: 10, 5, 1, 0.1, and 0.01mg·g^{-1} of food, mixed thoroughly into the pollen food described above. All of the PIs used were of comparable purity.

These four PIs were chosen as each is a candidate for incorporation into crop plant species and BPTI, SBTI and POT-2 have been expressed at insecticidal levels in transgenic plants [10, 15, 18, 19, 24]. The dosage levels used were equivalent to 4, 2, 0.4, and 0.04% of total protein and were chosen to cover and, in the case of the top dose, to exceed the range of expression levels that might be expected in the leaves of pest-resistant PI-transgenic plants [10, 15, 18, 19, 24]. The PI dosage levels chosen for bumblebees in the present study also allowed for comparison with similar studies on honeybees [4, 16, 17].

Four control cages with unadulterated pollen food were also set up. Three blocks of the experiment were set up on three separate occasions over four months: 4 PIs × 6 dosages × 3 blocks (72 cages in total). The bees were maintained and checked as above and their survival times determined.

To compare the effects of the various caging regimes or PI treatments on bee survival, the number of surviving bees was plotted against days from the beginning of the experiment for each cage of bees. These curves were then compared using Kayden-Meir estimates of survival distribution, S(t), and Mantel-Haenzel (log-rank) tests [14] . Both large and small bees were affected similarly by each PI and so these data were combined for final analysis (Figs. 1 to 4). Median survival times were also calculated and compared for the bees in each cage (Tabs. I, III). Mean rates of consumption of pollen, syrup and water were calculated (Tab. II) and compared using analysis of variance. For each experiment, data from the three blocks were combined as there were no significant block effects.

For the in vitro assays of proteolytic enzyme activity and inhibition, eight pooled extracts were prepared, each containing the guts of 12 bumblebees of various sizes. Pooled extracts were used to provide sufficient material for one complete set of inhibition assays to be carried out on each extract. Adult bees were taken directly from the colony, cold-anaesthetised and dissected to excise their midguts which were then frozen immediately in liquid nitrogen. These frozen guts were pooled into groups of 12 and weighed prior to extraction. The pooled samples were homogenised in 0.1 M Tris HCl pH 6.6 at 4 °C, extracting in 250 µL buffer per gut, and centrifuged at 15 870·g for 10 min at 4 °C to remove particulate matter. This pH was chosen to approximate bumblebee gut pH (unpublished observations).

Table I. Median survival times in days for large and small bumblebees kept singly or in groups of 5, 10 or 20. Values without a letter in common differ significantly at the 5% level.

Size of bees	Number of bees per cage			
	1	5	10	20
Large	47a	73 ab	68 ab	50 ab
Small	43 a	57 ab	94 b	95 b

Twenty five μL aliquots of extract were used in assays to determine the activities of three endopeptidases (elastase-like, chymotrypsin and trypsin) and one exopeptidase (leucine aminopeptidase (LAP)), as described by [5, 6]. The substrates (and final concentrations) used to assay these protease activities were N-Succinyl-L-Ala-L-Ala-L-Pro-L-Leu P-nitroanilide (SAAPLpNA, 0.52 μM), N-Benzoyl-L-Tyr P-nitroanilide (BTpNA, 1.25 μM), Nα-Benzoyl-DL-Arg P-nitroanilide (BApNA, 1.00 μM), and L-Leu P-nitroanilide (LpNA, 0.56 μM), respectively (all obtained from Sigma). The in vitro effects of BPTI, SBTI, POT-1 and POT-2 on each of these activities were determined by measuring protease levels in gut preparations after incubation with each inhibitor at 0.5, 1.0 and 2.0 μM (only 1.0 and 2.0 μM for BPTI). Enzyme and inhibitor (or buffer, in the case of controls) were preincubated for at least 5 min at 30 °C before the addition of the substrate. All protease assays were conducted, in duplicate, in 0.1 M Tris HCl pH 6.6 at 30 °C. Analysis of variance was used to compare the mean activities of the four proteases before and after treatment with the four inhibitors.

3. RESULTS

Survival data for small and large bumblebees kept singly or in groups of 5, 10 or 20 and supplied with pollen food, sugar syrup and water are summarised in Table I. Log-rank tests to compare the survival curves for bees kept under each of these regimes showed that large bees kept in groups of 20 were significantly shorter-lived than small bees in groups of 20 ($P < 0.001$), but that there were no significant differences in longevity between large and small bees for the other groupings. Among the small bees there were no significant differences in longevity under the different caging regimes ($P \leq 0.05$). Among the large bees, however, those kept in groups of 5 or 10 lived significantly longer than those kept individually or in groups of 20 ($P = 0.001$).

As pollen food, sugar syrup and water were consumed at reasonably steady rates throughout the bees' lifetimes, mean consumption rates over each bee's lifetime were calculated and compared (Tab. II). Mean pollen consumption rates (mg per bee per day) for small bees kept singly were significantly higher than those of small or large bees kept in groups of 5, 10 or 20 ($P \leq 0.05$). Large bees kept singly consumed significantly more pollen per day than large bees kept in groups of 10 or 20 or small bees in groups of 20 ($P \leq 0.05$). Daily rates of sugar syrup consumption were significantly higher for large bees kept in groups of 5 or 10 than for all groupings of small bees or groups of 20 large bees ($P \leq 0.05$). Large bees kept in groups of 10 also consumed significantly more syrup than large bees kept singly ($P \leq 0.05$). Water "consumption" varied greatly from day to day, but was always at a very low level compared with syrup consumption. Single large bees consumed significantly more water than any other category ($P \leq 0.05$).

Table II. Mean rates of consumption of pollen food, sugar syrup and water for large and small bumblebees kept singly or in groups of 5, 10 or 20. Values without a letter in common differ significantly at the 5% level, within each food type.

Type of food	Size of bees	Number of bees per cage			
		1	5	10	20
Pollen food	Small	35.3 a	9.7 bc	11.0 bc	5.3 b
(mg/bee/day)	Large	25.7ac	14.7 bc	8.7 b	3.3 b
Sugar syrup	Small	132.7 a	140.7 a	131.0 a	132.7 a
(μL/bee/day)	Large	172.7 ab	215.3 bc	233.0 c	125.7 a
Water	Small	19.7 a	12.0 a	7.3 a	5.0 a
(μL/bee/day)	Large	39.3 b	6.7 a	6.7 a	12.7 a

Survival curves for bees fed with PIs are shown in Figures 1 to 4 and their median survival times in Table III. Log-rank tests to compare survival curves indicated that there were significant differences in longevity among bees fed different doses of SBTI ($P < 0.001$), POT-1 ($P < 0.001$), and POT-2 ($P < 0.001$) (small and large bees combined) (Figs. 2–4). However, there were no significant longevity differences among bees fed different doses of BPTI and their controls (Fig. 1 and Tab. III).

Analysis of median survival times showed that there were no consistent patterns of response to different doses of SBTI, POT-1 or POT-2 (Tab. III). Bees fed 10 mg·g^{-1} SBTI had significantly poorer survival than the controls or those fed 1, 0.1 or 0.01 mg·g^{-1} ($P \leq 0.05$). Those receiving 5 mg·g^{-1} SBTI had median survival times that did not differ significantly from any of the other bees in the SBTI trial. Bumblebees had a partially dose-dependent survival response to POT-1. Bees that received 10 mg·g^{-1} POT-1 had significantly shorter lives than those fed 5 mg·g^{-1}, and these were significantly shorter-lived than the control bees ($P \leq 0.05$). The lifespans of bees receiving 1, 0.1 or 0.01 mg·g^{-1} POT-1 were intermediate between, and did not differ significantly from, the controls and the bees fed 5 mg·g^{-1} ($P \leq 0.05$). Bees fed 0.01 or 0.1 mg·g^{-1}

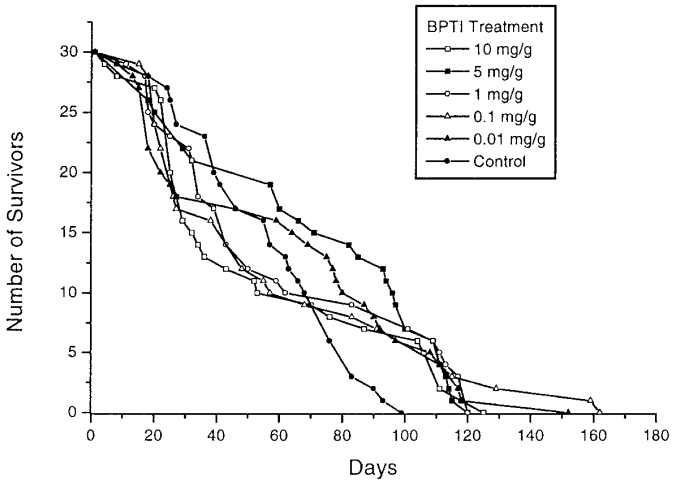

Figure 1. Survival curves for bumblebees fed the protease inhibitor, BPTI, in pollen food at five different dosage levels. Thirty bees are represented by each line; 3 replicates × 5 large and 5 small bees per cage combined.

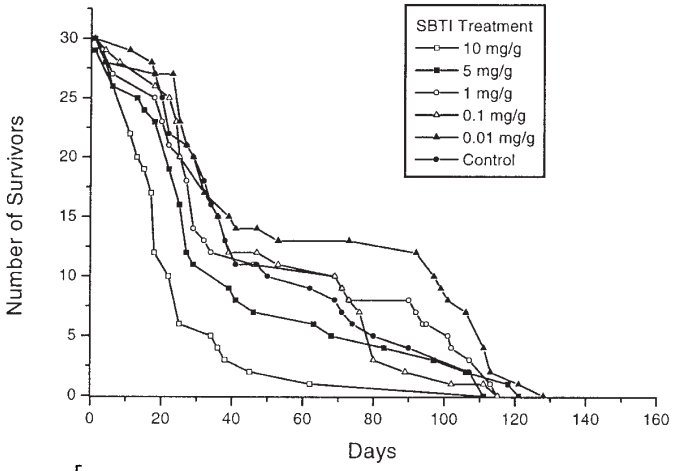

Figure 2. Survival curves for bumblebees fed the protease inhibitor, SBTI, in pollen food at five different dosage levels. Thirty bees are represented by each line; 3 replicates × 5 large and 5 small bees per cage combined.

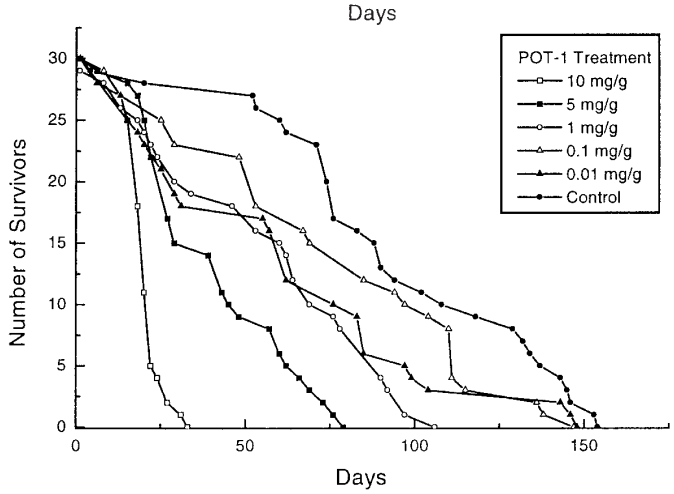

Figure 3. Survival curves for bumblebees fed the protease inhibitor, POT-1, in pollen food at five different dosage levels. Thirty bees are represented by each line; 3 replicates × 5 large and 5 small bees per cage combined.

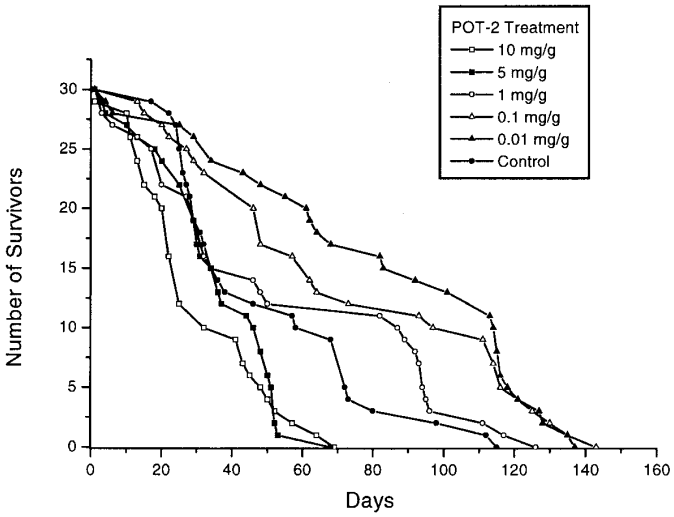

Figure 4. Survival curves for bumblebees fed the protease inhibitor, POT-2, in pollen food at five different dosage levels. Thirty bees are represented by each line; 3 replicates × 5 large and 5 small bees per cage combined.

Table III. Median survival times in days for bumblebees fed with protease inhibitors (PIs) added to pollen food at different dosage levels. Results from three replicates have been combined for each, i.e. each figure is derived from 15 small, 15 large or 30 bees of combined sizes. Treatments without a letter in common (across a row) have 95% confidence intervals (in parentheses) which do not overlap.

PI	Bee size	10 mg·g^{-1} PI	5 mg·g^{-1} PI	1 mg·g^{-1} PI	0.1 mg·g^{-1} PI	0.01 mg·g^{-1} PI	Control
BPTI	Small	34 (25-111)	71 (60-119)	43 (31-118)	27 (22-111)	69 (22-108)	62 (27-83)
	Large	32 (25-106)	85 (29-113)	50 (34-109)	55 (25-111)	59 (22-117)	57 (46-76)
	Combined	32 (25-76)	82 (57-100)	43 (34-101)	48 (25-83)	66.5 (25-90)	57 (41-73)
SBTI	Small	18 (15-25)	22 (22-83)	25 (20-92)	32 (25-73)	41 (25-111)	27 (22-90)
	Large	18 (13-36)	39 (27-106)	34 (29-107)	39 (36-89)	39 (32-113)	41 (36-80)
	Combined	18 (15-25) a	27 (25-41) ab	29 (27-73) b	39 (32-73) b	39 (32-101) b	38 (32-69) b
POT-1	Small	20 (18-22) a	29 (22-60) ab	56 (46-89) b	53 (29-101) b	31 (25-84) b	74 (59-108) b
	Large	20 (18-27) a	39 (27-66) ab	63 (29-92) b	104 (67-133) b	62 (55-143) b	118 (90-143) b
	Combined	20 (18-22) a	29 (27-57) b	61 (46-78) bc	83 (53-109) bc	62 (29-84) bc	88 (75-129) c
POT-2	Small	22 (20-52)	34 (27-52)	34 (29-94)	57 (46-116)	61 (43-92)	32 (27-78)
	Large	25 (22-50) a	36 (29-52) a	48 (29-96) a	64 (48-125) ab	116 (114-128) b	57 (32-80) a
	Combined	25 (22-43) a	35 (29-50) ab	40 (29-92) ab	62 (48-114) b	87.5 (62-115) b	36 (29-73) ab

POT-2 lived significantly longer than those fed 10 mg·g^{-1} of this PI ($P \leq 0.05$). However, the control bees and those that received 5 or 1 mg·g^{-1} of POT-2 had intermediate survival times that did not differ significantly from those of bees receiving the highest and lowest doses of this PI.

Protease levels in preparations made from untreated bumblebee midguts were as follows: elastase-like (SAAPLpNA-digesting), 283.0 ± 16.9 nmol·min^{-1}·g^{-1} of gut; chymotrypsin (BTpNA-digesting), 148.5 ± 8.4 nmol·min^{-1}·g^{-1} of gut; trypsin (BApNA-digesting), 27.2 ± 2.8 nmol·min^{-1}·g^{-1} of gut; LAP (LpNA-digesting), 258.6 ± 9.6 nmol·min^{-1}·g^{-1} of gut (data from eight pooled extracts). The effects of adding the four different inhibitors (BPTI, SBTI, POT-1 and POT-2) to these gut preparations are shown in Figure 5. As expected, the exopeptidase, LAP, was unaffected by these four endopeptidase inhibitors. The elastase-like activity, which appears to be an important endopeptidase activity in bumblebee guts, was strongly inhibited by each concentration of POT-1 and POT-2 ($P < 0.001$). SBTI also significantly inhibited this activity, in a dose-dependent fashion ($P < 0.001$). BPTI had no significant inhibitory effect. Chymotrypsin was also strongly inhibited by each concentration of POT-1 and POT-2 ($P < 0.001$), inhibited significantly by only the highest concentration of SBTI ($P = 0.001$), and not at all by BPTI. Trypsin levels were very low and were significantly inhibited by all the inhibitors tested ($P < 0.001$).

4. DISCUSSION

The results of the first experiment to determine the survival and food/drink consumption of bees of different sizes under different grouping regimes suggested that small bees have greatest longevity in groups of 20, but that large bees survive best in groups of 5 or 10. Single bees, both large and small, had the shortest survival times (significantly so in the case of the large bees). This result was not unexpected as other social insects, such as worker honeybees (*Apis mellifera*), have poor survival if kept in groups of less than about 20 (unpublished observations). That *B. terrestris* can tolerate being kept with as few as four companions may reflect the less rigid social structure of this insect compared with that of the honeybee.

This experiment also showed that daily pollen consumption did not vary significantly over the lifetime of each bumblebee. This contrasts with the honeybee, where most protein consumption occurs during the first few days of adult life and then drops steadily to very low levels [7]. This need for protein is related to the development of the hypopharyngeal glands which honeybees undertaking nursing duties need in order to secrete "brood food" for their larvae [26]. Bumblebees do not have such a strict division of labour, their glands do not vary in size [12] and therefore their protein requirement throughout adult life probably does not vary much. Even though the bumblebees in this experiment were caged and thus prevented from undertaking their normal duties, the steady pollen consumption rates observed here suggest an unchanging requirement for protein food by adult bumblebees. However, because the ages of the bumblebees at the start of this experiment were not known, but ranged from newly-emerged to about eight weeks old (according to the supplier), there is still a possibility that these insects also have an early peak which was missed in every case, although this seems unlikely. For both small and large bumblebees, the highest pollen consumption rates were observed for bees kept singly and the lowest rates for those kept under the most crowded conditions, 20 per cage. This may have been because the single bees had no competitors for their food (i.e. they "overate"), and the crowded bees were actually limited by the quantities of food supplied (i.e. somewhat starved).

In cages, sugar syrup was also consumed at a steady rate throughout each bumble-

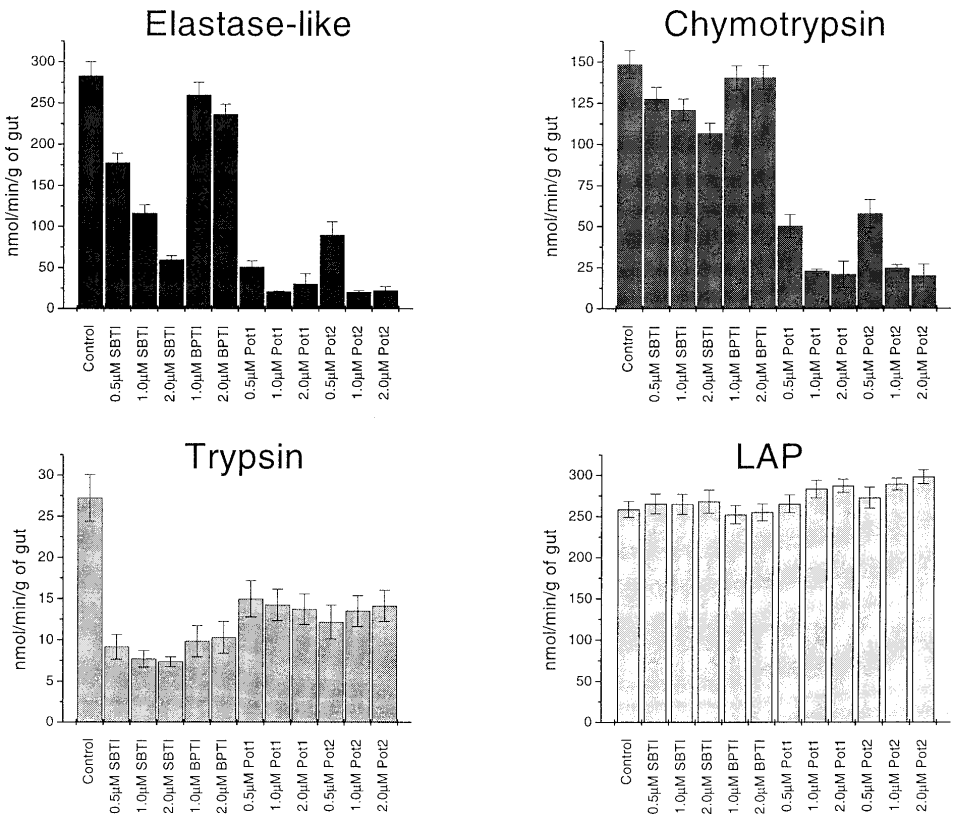

Figure 5. Inhibition of four protease activities, elastase-like, chymotrypsin, trypsin and leucine aminopeptidase (LAP), in preparations made from untreated bumblebee midguts by four protease inhibitors, SBTI, BPTI, POT-1 and POT-2, at a range of concentrations. Mean activity levels and their standard errors are shown. Duplicate assays were conducted for each of eight gut preparations, each consisting of 12 bee guts.

bee's lifetime. Under more realistic conditions, variations in activity levels among bees carrying out foraging or housekeeping activities in the field may create differing demands for carbohydrate food. Large bees kept in groups of 5 or 10 had the highest rates of syrup consumption. This is difficult to explain. Their large size may have meant they had a greater carbohydrate requirement than the small bees, but this does not explain why they drank more than the other groupings of large bees. Perhaps the social interactions among the groups of 5 and 10 bees created an energy demand. Groups of 20 large bees may have had their consumption limited by the supply, but single bees would not have had this difficulty.

Water was consumed in only small amounts and only the large single bees consumed significantly greater quantities than the others. This was not surprising as bumblebees have not been observed to collect water in the field as honeybees do [12] . The "consumption" observed here may have in fact been the result of bees erroneously drinking from the water feeders instead of the syrup feeders. In olfactory learning experiments, bumblebees do not perform as

well as honeybees (M.H. Pham-Delegue, personal communication) and so they may have repeatedly made such errors.

In terms of the preferred bioassay criteria, using single bees would have allowed for the most degrees of freedom, but because their pollen consumption was higher and their survival not as great as that of the bees kept in groups, it was decided that a grouping of 5 large and 5 small bees placed together would be the best regime for the PI experiments. With this arrangement, reasonable survival times could be expected, both sizes of bees could be assessed and PI usage (and thus cost) would not be excessive.

Bumblebee survival was not significantly affected by BPTI. Responses to SBTI, POT-1 and POT-2 did not follow any consistent pattern, except that the highest PI dose resulted in the lowest survival in each case. All other doses of SBTI and POT-2 resulted in survival times that were indistinguishable from those of their control bees. Only in the POT-1 trial was there some evidence of a dose-dependent response to PI-feeding, as bees given 5mg·g^{-1} POT-1 had poorer survival than their control bees, but better than those receiving 10 mg·g^{-1} of this PI. Control bee survival times varied considerably from one trial to another, perhaps because of the unknown ages of the bees at the start of the experiment, and this may have made trends in dose-response harder to detect. However, the complete lack of variation in response to different doses of BPTI suggests strongly that bumblebee survival is not affected at all by this PI.

These results contrast with those obtained in similar experiments with the honeybee, where adult bee survival is reduced in a dose-dependent fashion by each of these four inhibitors [4, 16, 17]. The different patterns of protein use in the two species may explain this. Disruption of the adult honeybee's early, critical phase of high protein consumption by an inhibitor may significantly lower bee longevity. In contrast, the steady rates of protein consumption displayed by bumblebees, apparently utilising quite low levels of proteases, suggest that they do not have a critical phase of protein consumption. Thus the effects of protease inhibition may not be as significant in terms of effects on bumblebee survival, unless the PI strongly and specifically inhibits a particularly important protease or proteases. In vitro assays showed that POT-1, POT-2 and SBTI strongly inhibited elastase-like activity, which appears to be an important endopeptidase activity in the bumblebee midgut. All concentrations of POT-1 and POT-2 and the highest concentration of SBTI also inhibited chymotrypsin activity. Lower concentrations of SBTI did not significantly inhibit chymotrypsin activity. The relative importance of elastase-like and chymotrypsin activities in the bumblebee digestive system and their relative insensitivity to BPTI may explain why bees fed high doses of POT-1, POT-2 and SBTI have poor survival.

The concentrations of PIs used in this experiment (10, 5, 1, 0.1 and 0.01 mg·g^{-1} pollen-food) are equivalent to 4, 2, 0.4, 0.04 and 0.004% of total protein. Although pollen expression levels have not yet been recorded, pest-resistant PI-transgenic plants with leaf expression levels ranging from 0.05 to 2.5% have been shown to be protected against insect attack. For example, transgenic rice plants expressing a POT-2 gene at 0.5 to 2% of total soluble protein have been shown to effectively control pink stem borer [10] and transgenic tobacco expressing 0.22 to 0.65% POT-2 significantly reduces the growth of larval green loopers [19]. SBTI-tobacco plants have been shown to reduce the growth of *Spodoptera litura* larvae when expressing SBTI at 0.2 to 0.4% of total protein and to kill this insect at 0.4 to 1% [18]. Transgenic rice expressing 0.05 to 2.5% SBTI is resistant to brown planthopper [15]. Transgenic white clover plants expressing 0.07% BPTI significantly reduce the growth of the pasture pest lepidopteran, *Wiseana cervinata* [24]. Thus,

bumblebee survival is unlikely to be affected by transgenic plants expressing the levels of SBTI, POT-1 or POT-2 needed for pest control, and plants expressing BPTI at even 4% of total protein would not be expected to be toxic to bumblebees. Furthermore, if PI genes were engineered into plants within constructs that did not allow expression in pollen or nectar, then bees would not be exposed to these proteins at all.

We have established that small cage trials can be conducted with bumblebees to test pest-resistance gene products and conclude from these trials that bumblebees are less likely to be affected by transgenic plants expressing PIs than honeybees.

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Résumé – Effets de quatre inhibiteurs de protéases sur la longévité des ouvrières de bourdons, *Bombus terrestris* L. Un test biologique a été mis au point sur des bourdons adultes pour étudier les effets de produits de gènes qui confèrent une résistance aux ravageurs. Ce test a permis de déterminer l'action de quatre inhibiteurs de protéases (IP)sur la longévité des bourdons ; il s'agit de l'inhibiteur de trypsine soja de Kunitz (SBTI), l'inhibiteur de trypsine pancréatique bovine (BPTI), des inhibiteurs de protéases de pomme de terre (POT-1 et POT-2).

Des ouvrières de petite et de grande taille ont été maintenues individuellement ou en groupes de 5, 10 ou 20 individus dans des cages à l'étuve et leur survie et leur taux de consommation de pollen, de sirop et d'eau ont été comparés.

La durée moyenne de survie a été comprise entre 43 et 95 jours. Les gros bourdons en groupes de 20 ont vécu moins longtemps que les petits en groupes de 20 ($P < 0{,}001$), mais il n'y a pas eu de différence significative entre les gros bourdons et les petits dans les autres groupes. Parmi les petits bourdons il n'y a pas eu de différence de longévité en fonction du nombre d'individus par groupe ($P \leq 0{,}05$). Mais parmi les gros bourdons, ceux qui étaient groupés par cinq ou dix ont vécu plus longtemps que les isolés ou ceux en groupes de 20 ($P = 0{,}001$). Le pollen et le sirop ont été consommés régulièrement tout au long de la vie (3,3–35,3 mg de pollen par bourdon et par jour) et 125,7–233,0 µL par bourdon et par jour). L'eau a été consommée régulièrement et en quantité négligeable (5,0–39,3 µL par bourdon et par jour). Les petits bourdons maintenus individuellement ont consommé en moyenne plus de pollen que les petits ou les gros bourdons en groupes de 5, 10 ou 20 ($P \leq 0{,}05$) et les gros bourdons maintenus individuellement en ont consommé plus que les gros bourdons en groupes de 5, 10 ou 20 ($P \leq 0{,}05$).

La consommation journalière de sirop a été plus élevée chez les gros bourdons en groupes de 5 ou 10 que pour ceux groupés par 20 ou que pour tous les petits bourdons ($P \leq 0{,}05$) et celle des gros bourdons groupés par 10 supérieure à celle des bourdons maintenus individuellement ($P \leq 0{,}05$).

Des quatre inhibiteurs, seul le BPTI n'a pas eu d'effet significatif sur la longévité des bourdons (Fig. 1). La longévité des bourdons ayant reçu 10 $mg \cdot g^{-1}$ de SBTI (Fig. 2), de POT-1 (Fig. 3) ou de POT-2 (Fig. 4) a été significativement plus courte que celle des témoins ($P \leq 0{,}05$). Les bourdons n'ont pas répondu de façon homogène aux autres doses (0,01; 0,1; 1 et 10 $mg \cdot g^{-1}$) de chacun des IP. Seuls les résultats du POT-1 suggèrent une réponse liée à la dose. Les bourdons qui ont reçu 5 mg/g de POT-1 ont vécu plus longtemps que ceux qui en ont reçu 10 $mg \cdot g^{-1}$, mais moins longtemps que ceux qui ont reçu les autres dose et que les témoins (Tab. III).

L'activité protéasique/protéolytique par intestin(nmol·min^{-1}·g^{-1}) a été mesurée sur des préparations d'intestins de bourdons non traités (Fig. 5). Les valeurs sont les suivantes : enzyme semblable à l'élastase : 283,0 ± 16,9 ; chymotrypsine 148,5 ± 8,4 ; trypsine 27,2 ± 2,8 ; leucine aminopeptidase 258,6 ± 9,6. Dans les test d'inhibition in vitro, les activités de l'enzyme semblable à l'élastase et de la chymotripsine ont été significativement plus réduites par SBTI, POT-1 et POT-2 mais pas du tout par BPTI. Nous concluons qu'il est possible de faire des essais en cagettes avec les bourdons pour tester les produits de gènes exprimant une résistance aux ravageurs et que les bourdons sont moins susceptibles d'être affectés par les plantes transgéniques exprimant des inhibiteurs de protéases que les abeilles.

***Bombus* / inhibiteur de protéases / consommation alimentaire / longévité / plante transgénique résistante aux ravageurs / test en cagette**

Zusammenfassung – Wirkung von vier Proteasehemmern auf die Überlebensrate von Hummelarbeiterinnen, *Bombus terrestris* L. Ein Biotest mit adulten Hummeln wurde entwickelt, um Auswirkungen von transgenen Produkten zu untersuchen, die eine Schädlingsresistenz bewirken. Mit diesem Test wurde die Wirkung von 4 verschiedenen Substanzen, die eiweißabbauende Enzyme hemmen, auf die Überlebensrate von Hummeln bestimmt. Es wurden der Kunitz Trypsin Inhibitor aus Sojabohnen (SBTI), der Pankreas Trypsin Inhibitor aus Rindern (BPTI) und die Protease-Inhibitoren aus Kartoffeln 1 (POT-1) und 2 (POT-2) untersucht.

Kleine und große Arbeiterinnen wurden einzeln oder in Gruppen mit 5,10 und 20 Hummeln in Käfigen im Brutschrank gehalten. Ihr Überleben und die Menge des verbrauchten Pollen, Zuckerwassers und Wassers wurde verglichen. Die Überlebenszeit betrug im Mittel 43–95 Tage. Große Hummeln, die in Gruppen von 20 gehalten wurden, lebten kürzer als kleine Hummeln in gleicher Gruppenstärke. ($P < 0{,}001$), aber es gab keine signifikanten Unterschiede in der Lebensdauer zwischen großen und kleinen Hummeln in den anderen Gruppen. Bei den kleinen Hummeln gab es bei unterschiedlichen Käfigbedingungen keine Unterschiede in der Lebensdauer ($P \leq 0{,}05$). Bei den großen Hummeln dagegen lebten die Gruppen mit 5 oder 10 Tieren länger als Einzeltiere oder 20er Gruppen ($P = 0{,}001$). Pollen und Zuckersirup wurden während der gesamten Lebensdauer in gleichmäßiger Menge verbraucht (3,3–35,3 mg Pollen pro Hummel und Tag, 125,7–233,0 µL Sirup pro Hummel und Tag). Wasser wurde ebenfalls gleichmäßig aufgenommen, wenn auch nur in geringen Mengen (5,0–39,3 µL pro Hummel und Tag). Wurden kleine Hummeln einzeln gehalten, war der Pollenverbrauch höher als bei kleinen und großen Hummeln, die in Gruppen mit 5, 10, oder 20 Tieren gehalten wurden ($P \leq 0{,}05$). Einzeln gehaltene große Hummeln verbrauchten mehr Pollen pro Tag als große in Gruppen gehaltene Tiere (5 oder 10) bzw. als kleine Tiere in 20er Gruppen ($P \leq 0{,}05$). Große Tiere in 5er bzw. 20er Gruppen verbrauchten mehr Zuckerwasser als alle anderen Gruppen mit kleinen oder den Gruppen mit 20 großen Tieren ($P \leq 0{,}05$). Große Hummeln der 10er Gruppen verbrauchten mehr Zuckerwasser als wenn sie einzeln gehalten wurden ($P \leq 0{,}05$).

Von den 4 Protease-Hemmern hatte nur BPTI keine signifikante Wirkung auf die Lebensdauer (Abb. 1). Hummeln, die 10 mg·g^{-1} SBTI (Abb. 2), POT-1 (Abb. 3) oder POT-2 (Abb. 4) aufnahmen, lebten siginifikant kürzer als die Kontrolltiere ($P \leq 0{,}05$). Es gab kein einheitliches Reaktionsmuster bei anderen Dosen der Protease-Inhibitoren. Nur die Ergebnisse mit POT-1 wiesen auf eine dosisabhängige Reaktion hin. Nach Fütterung von 5 mg·g^{-1} POT-1 lebten die Hummeln länger als nach Fütterung von 10 mg·g^{-1} aber kürzer als nach anderen Dosen bzw. als die Kontrollen (Tab. III).

Unbehandelte Hummeln wurden präpariert und die Protease Aktivität (nmol·min^{-1}·g^{-1}) wurde pro Darm bestimmt (Abb. 5). Die Werte betrugen bei dem Elastase ähnlichen Enzym 283,0 ± 16,9, bei Chymotrypsin 148,5 ± 8,4, bei Trypsin 27,2 ± 2,8 und bei Leucin Aminopeptidase 258,6 ± 9,6. Bei Hemmversuchen in vitro war die Aktivität des Elastase ähnlichen Enzyms und des Chymotrypsins signifikant durch SBTI, POT-1 und POT-2, aber nicht durch BPTI reduziert. Wir schlieβen aus diesen Versuchen, dass Teste in kleinen Käfigen mit Hummeln geeignet sind, um die Wirkung von transgenen Produkten zu untersuchen, die eine Schädlingsresistenz bewirken. Auch scheint die Wahrscheinlichkeit einer Schädigung von Hummeln durch transgene Pflanzen mit Hemmung der Protease Aktivität geringer ist eine Schädigung von Honigbienen.

Hummeln / Protease Inhibitoren / Futterverbrauch / Biotest im kleinen Käfig / schädlingsresistente transgene Pflanzen

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