Inhibition of Larval Growth in the Gypsy Moth (Lepidoptera: Lymantriidae) by Venom from the Parasitic Wasp *Microbracon hebetor* (Hymenoptera: Braconidae)[1]

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Abstract Extract of the venom glands of the adult female ectoparasitic wasp *Microbracon hebetor* (Say) (Hymenoptera: Braconidae) inhibited larval growth in the gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae). The inhibition was dose dependent and at 5 d after injection larval growth was reduced 50% by 2×10^{-3} µg venom gland protein per mg larval weight. Doses greater than 2.5×10^{-2} µg per mg larval weight prevented growth completely and accelerated larval death. Size-exclusion fractions of gland extract, which eluted between 35 kD and 66 kD molecular weight markers, inhibited growth and caused delayed pupation. At 5 d after injection of active chromatographic fractions, larval growth was reduced 50% by 2×10^{-4} µg fraction protein per mg larval weight. Doses greater than 1×10^{-3} µg fraction protein per mg larval weight stopped growth completely. Both crude extract and active column fractions caused their effects without evidence of extended paralysis.

Key Words Chromatography, growth inhibition, larval mortality, *Lymantria dispar, Microbracon hebetor,* pupation delay, wasp venom.

Parasitic wasps employ a variety of complex metabolic and physiological interactions with their insect hosts to ensure the survival and maturation of the parasitoid larvae (Alleyne et al. 1997, Godfray 1994). Because the ectoparasitic wasp depends upon the host as a site for larval parasitoid development, rapid death of the host is not a primary goal, and a reduction of host movement and molting is advantageous. Thus, the roles of venom components of ectoparasitic wasps are of special interest because they suppress host development following stinging (Coudron et al. 1990, Doury et al. 1995). Although venom components can be fatal, sublethal doses exhibit various physiological and developmental effects including paralysis and molt inhibition (Coudron et al. 1990, Piek et al. 1982). Venom of the ectoparasitic wasp *Microbracon hebetor* (Say) (Hymenoptera: Braconidae) has been of considerable interest because it is quite potent and the wasp has a rather wide host range in the Lepidoptera (Beard 1952, Drenth 1974, Piek 1966, Piek et al. 1982, Quistad et al. 1994, Visser et al. 1983). Biochemical analyses have focused on its paralytic properties (Drenth 1974, Piek et al. 1982, Spanjer et al. 1977, Visser et al. 1976, 1983). Three protein toxins

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with molecular weights from 18 kD to 57 kD, exhibiting neuromuscular and paralytic activities in lepidopteran larvae, have been isolated from *M. hebetor* venom (Slavnova et al. 1987, Visser et al. 1983). Three additional protein toxins with molecular weights near 73 kD have been purified and partially sequenced (Quistad et al. 1994). These toxins have acute lethal effects, killing a variety of lepidopteran larvae within 24 h of injection. In a search for biologically-derived factors for the control of the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), we explored the reservoir of biological agents in the venom of *M. hebetor.* We now report a demonstration of developmental changes in *L. dispar* larvae injected with *M. hebetor* venom, and the discovery of a protein component of venom, in the molecular weight range of 35 kD-66 kD, which retards larval growth and delays pupation.

Materials and Methods

Animal rearing and maintenance. The *L. dispar* culture used in this study originated at the USDA/APHIS Methods Development Laboratory, Otis AFB, MA [strain NJ-SS; O'Dell et al. 1984] and was maintained according to established methods (Bell et al. 1981). Neonates were reared on a high wheat-germ diet (Bell et al. 1981), 10 larvae per 200-ml plastic cup. Larvae were held at 25°C, 50 to 60% relative humidity, a 16hL:8hD photoperiod (Masler et al. 1991) and collected prior to the second larval molt (from the second to the third larval stadia) for use in experiments. Depending upon the experiment, larvae were maintained in the 200-ml cups at 5 to 8 larvae per cup, or transferred to 50-ml cups at 1 larva per cup. Under these conditions control males (n = 20) attained a maximum weight of 846 ± 194 mg (mean ± S.E.M.) 16 d after the molt to the third stadium. Control females (n = 35) reached a maximum weight of 2474 ± 80 mg 3 d later than males. After reaching maximum weight, larval shrinkage and weight loss occurred in both sexes in preparation for pupation. Male pupation typically began about 21 d after the start of the third stadium, and female pupation began 1 to 2 d later.

Gland dissection and extract preparation. *Microbracon hebetor* were obtained live from BioFac (Mathis, TX) and stored at –80°C. Frozen females were removed from the freezer and kept on ice just prior to dissection. Glands were dissected from adult female wasp abdomens, separated from attached tissues, and collected directly into 1.5-ml polypropylene tubes (Kontes Scientific, Vineland, NJ) on dry ice (50 glands per tube) and stored at –80°C until use. Extracts were prepared by homogenizing in the storage tube with a Teflon® pestle (Kontes Scientific, Vineland, NJ) by hand in *Bombyx* saline (Okuda et al. 1985, 2 glands/µl). Homogenates were centrifuged at 16,000 g for 3 min at room temperature. Supernatants were collected, stored on ice, and aliquots prepared for total protein estimation and for bioassay. Dilutions were prepared using *Bombyx* saline.

Chromatographic fractionation. Venom glands, collected as described, were extracted by homogenizing in batches of 50 glands in 100 µl of phosphate buffer (50 mM, pH 7.0, 300 mM NaCl), by hand, using the Teflon homogenizer-polypropylene tube combination described above. Extracts were centrifuged at 20,800 g for 3 min at room temperature. Typically, 200 glands were used for each chromatographic preparation. Pellets from the first centrifugation were re-extracted as above, and the supernatants added to the previous pool. The entire pool (both sets of extractions) was centrifuged at 48,000 g for 4 min at 4°C, and the supernatant collected. This high-speed supernatant was concentrated to approximately 50% of the starting volume

under vacuum (SpeedVac, Savant Instruments, Farmingdale, NY), then injected onto the chromatographic system. Fractionation was carried out on a TSK G3000SWxl high performance size-exclusion column (7.8 × 300 mm; TosoHaas, Montgomeryville, PA), with a mobile phase of 50 mM phosphate buffer, pH 7.0, 300 mM NaCl, and a flow rate of 0.5 ml/min. Dead volume was 5 ml (10 min after injection). Fractions (1 min = 0.5 ml each) were collected from 10 to 30 min after sample injection onto the column and stored on ice until bioassay. For bioassay, 50 µl of each fraction were diluted 1:1 with water, then injected (1.5 µl) into third-instar, day-1 larvae. Remaining fraction aliquots were stored at −20°C until further use. The column was calibrated with the following standards (Sigma, St. Louis, MO): thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD), bovine serum albumin (dimer, 132 kD), bovine serum albumin (monomer, 66 kD), egg albumin (45 kD), pepsin (35 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), lysozyme (14.3 kD).

Injection. Injections were made using an apparatus constructed with a 26-gauge needle (Beckton-Dickinson, Rutherford, NJ) attached to a 25-µl microsyringe (Hamilton, Reno, NV) via Teflon tubing (440 um i.d.). The microsyringe was mounted in a Hamilton stepper syringe holder and the system calibrated to deliver test solutions in 0.5 µl increments (1.5 µl = 3 clicks of the syringe adapter). The youngest larvae injected without excessive death due to injury were day-1 third instars, typically weighing 40 to 60 mg. For injection, larvae were anaesthetized by placing them under CO_2 vapor generated from small pieces of dry ice. Injected larvae were allowed to recover, then placed in food cups and returned to the rearing incubator. Larvae were observed daily, weighed, and mortality assessed by lack of movement when disturbed by picking up and moving with forceps. In some cases, larvae initially assessed as "dead" subsequently recovered and began to feed. Thus, even after larvae appeared to be dead, they were observed for up to 1 wk to avoid false scoring.

Protein estimation. Total protein in gland extracts and column fractions was estimated using the BioRad protein estimation kit (BioRad, Richmond, CA) following the manufacturer's recommendations for use in a 96-well microtiter plate format (polystyrene, flat-bottom, Corning Easy-Wash Assay Plates, Corning Plastics, Corning, NY). The plate reader filter was set to 560 nm.

Results and Discussion

Crude gland extract, growth inhibition and mortality. We examined the effect of *M. hebetor* venom gland extract on the development of *L. dispar* larvae and found significant developmental effects dependent upon the doses of venom gland extract injected. Crude *M. hebetor* venom gland extract was lethal to *L. dispar* larvae when applied at high doses and may have a paralytic effect, although *L. dispar* is less sensitive to the venom extract than many other lepidopterans (Drenth 1974). Injection of 1 µg of this protein per mg of *L. dispar* larval weight invariably lead to death within 24 h (Table 1). Injected larvae never recovered and were immobile. Injection of less than 1 µg venom gland protein per mg larval body weight did not induce extended paralysis but did lead to an increased, dose-responsive larval mortality. Some mortality was observed 2 d after injection with all but the lowest dose tested (Table 1). Most larvae surviving at least 24 h after injection were mobile and apparently feeding as evidenced by frass pellets in the rearing cups, but sub-lethal doses retarded larval growth. Fig. 1 shows that at 24 h after injection, larvae treated with 2.0×10^{-4} µg venom gland protein, or more, per mg larval weight weighed less than saline-injected

Table 1. Percent mortality of *L. dispar* larvae on days following injection with *M. hebetor* venom gland extract

Dose*	n**	Days after Injection			
		1	2	7	14
0.0005	21	0	0	0	5
0.001	39	0	10	8	22
0.1	8	0	50	50	88
0.8	9	0	78	78	100
1.0	16	100	—	—	—

Day 1, third-instar larvae were injected with 1.5 µl of venom gland extract at the indicated doses.
* µg venom gland protein per mg larval weight.
** number of larvae.

controls. The effect was dose-dependent, and at 2.5×10^{-2} µg venom gland protein per mg larval weight there was no weight gain above the larval weight at the time of injection. At 5 d after injection (Fig. 1), the dose-responsive suppression of growth was clear. Growth was completely suppressed by injection of 4.5×10^{-2} µg venom gland protein per mg larval weight. The ED_{50} (growth reduced 50% relative to controls) determined at 5 d after injection was 2×10^{-3} µg venom gland protein per mg larval weight.

Extract fractions, growth inhibition and mortality. Venom gland extract processed by size-exclusion chromatography yielded fractions which reduced weight gain when injected into day-1 third-instar larvae (Fig. 2). Growth inhibitory activity was present in a series of fractions eluting between 8 and 10 ml, with maximum activity eluting in fractions between the 35 kD and 66 kD molecular weight markers (Fig. 2). The most effective fractions (9-9.5 ml elution volume) were pooled and tested for growth and mortality effects.

At the highest fraction dose tested (3×10^{-3} µg fraction protein/mg larval weight), mortality was first observed at 5 d after injection (25%), increased at d 7 (50%) and reached 75% at 14 d after injection (n = 4 for all tests). This is in contrast to the acute toxicity of the 73 kD proteins isolated from *M. hebetor* (Brh toxins; Quistad et al. 1994) which kill a number of lepidopteran species within 24 h of injection. The active factor(s) in our preparations also differ from the Brh toxins in apparent molecular weight, but are similar in that they are labile when exposed to denaturing conditions. Although we recovered toxic activity after freeze-thawing, we could not recover activity using 40% acetonitrile in 0.1% trifluoroacetic acid.

A slight reduction of larval growth by column fraction was detected 24 h after injection and became more apparent on d 5 after injection (Fig. 3). Growth was reduced by all doses tested, and completely inhibited by injection of 1×10^{-3} µg fraction protein per mg larval weight. On d 7 after injection (Fig. 3), the dose response was quite pronounced, and growth remained completely inhibited at 1×10^{-3} µg fraction protein per mg larval weight. ED_{50} occurred at 2×10^{-4} and 4×10^{-4} µg fraction protein per mg larval weight for d 5 and d 7 respectively.

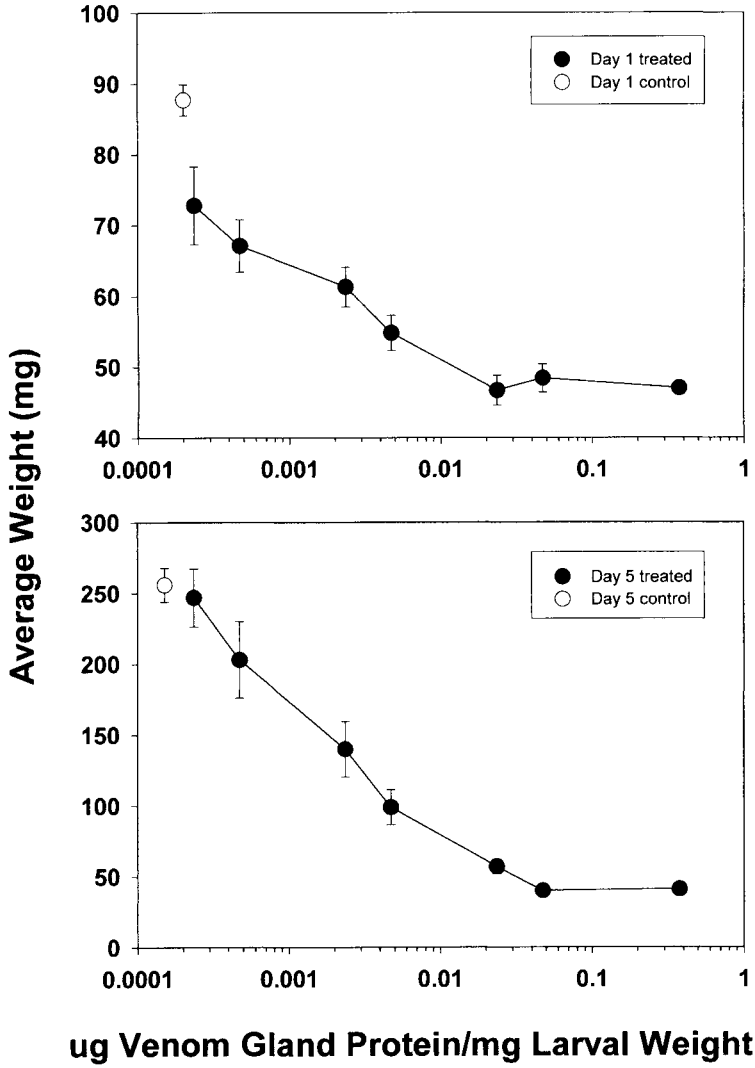

Fig. 1. Effect of venom gland extract on larval weight. Each data point represents the mean weight (mg) ± S.E.M. of 5 to 27 separate larvae.

Slowed development. Larvae which were treated with greater than 1×10^{-1} µg crude venom gland protein, or 1×10^{-3} µg fraction protein, per mg larval body weight, could survive for extended periods, but they gained little to no weight and died as larvae (Fig. 4). Larvae treated with lower doses of either preparation grew more slowly than controls, but typically survived to pupate. We further examined this effect using a sublethal dose of active fraction. Table 2 shows that after injection of 5×10^{-4} µg fraction protein per mg larval weight, pupation was delayed in both sexes, males

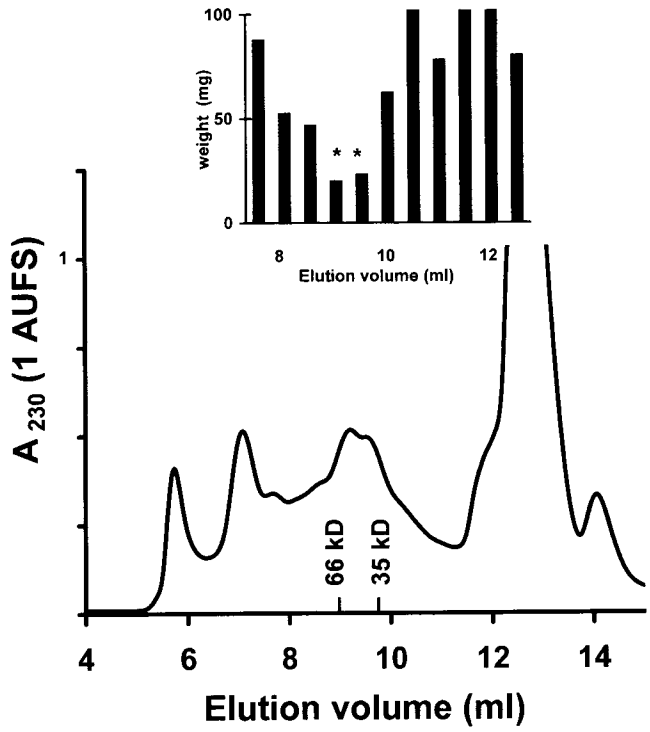

Fig. 2. Fractionation of venom gland extract by size-exclusion chromatography. Inset: Average weight of 3 larvae injected with fraction aliquot, 2 d after injection. *fractions used for further study.

pupated 4 to 5 d later than saline-injected controls, and females pupated 2 to 5 d later than controls. The differences in the mean day of pupation between treated and control animals were significant (Student's *t*-test, $P < 0.05$) for each sex. Although growth was slowed in larvae injected with the active column fraction, the mean weights for male pupae from treated and control animals were the same, and the mean weight for female pupae from treated larvae was higher (17%) than the mean weight of control pupae.

Venom glands of *M. hebetor* contain factors that caused mortality in *L. dispar* larvae and, at lower doses, a distinct inhibition of growth. During development of larvae treated with sub-lethal doses of extract, animals were mobile and showed a dose-dependent rate of growth. Because animals were mobile, feeding was not restricted due to paralysis. Whether feeding was restricted by some other mechanism, or metabolism was compromised, or both, remains to be determined. The growth inhibition was caused by one or more labile proteins within the molecular weight range of 35-66 kD. Unlike the paralytic (Slavnova et al. 1987, Spanjer et al. 1977, Visser et al. 1976) and lethal (Quistad et al. 1994) toxins reported in *M. hebetor,* the 35-66 kD proteins in our preparation inhibited growth, delayed pupation and, only at elevated doses, were lethal. Fatality appeared to be due to starvation and was not rapid.

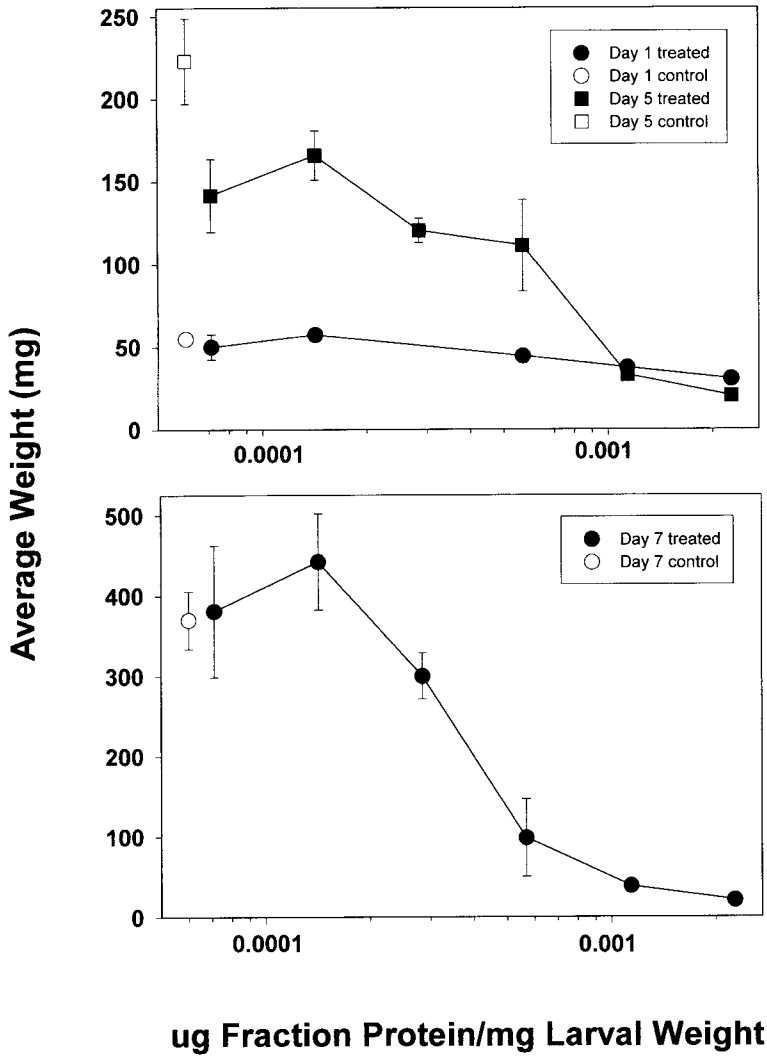

Fig. 3. Effect of active chromatographic fraction on larval weight. Each data point represents the mean weight (mg) ± S.E.M. of 3 to 9 separate larvae.

Such active factors should be of use in control strategies as supplements to the use of toxins or toxin analogs in, for example, transgenic plants. Long-term antifeedant activity, acting through a mechanism different from paralysis or acute toxicity, may prevent development of individuals resistant to those other treatments, and also may reduce the levels of toxins necessary for population control. The effect of the growth-inhibiting factors from *M. hebetor* on lepidopterans other than *L. dispar* is an important issue to address given species variation in response to toxins and paralytics

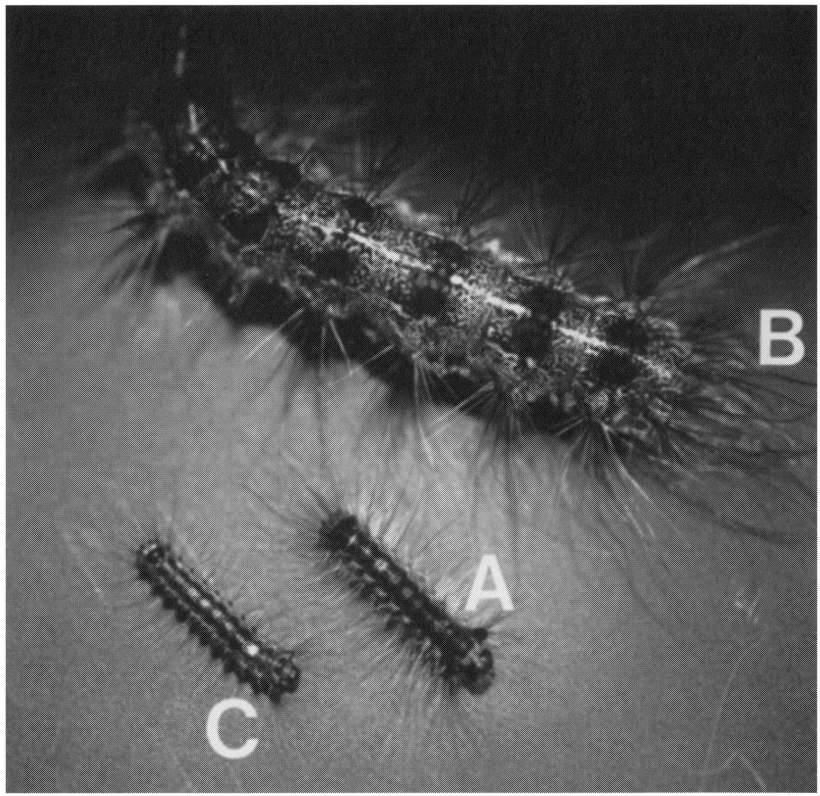

Fig. 4. Inhibition of larval growth by injection of active chromatographic fraction. Larva A injected with active fraction and larva B injected with inactive fraction, on day 1 of third larval instar; observed 18 days after injection. Larva C, normal day 1 third instar.

(Drenth 1974, Quistad et al. 1994). Biologicals such as natural toxins and growth inhibitors represent a largely untapped reservoir of control potential. Indeed, less than 10% of all insecticides now in use involve biologicals (Casida and Quistad 1998). There is a pressing need for further examination of biologically-derived pest control agents in light of both environmental and economic concerns.

Another potentially important application of growth inhibitors is in the study of *L. dispar* (and other lepidopteran) development and physiology. Natural molecular probes affecting specific metabolic or physiological processes may be used to discover or identify developmental events important for survival, and thus potential targets for control (Jones and Coudron 1993). For example, non-paralytic components of the venom of the ectoparasite *Euplectrus plathypenae* (Howard) (Hymenoptera: Eulophidae) block molting in *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) while allowing the larva to feed and grow (Coudron 1991, Coudron et al. 1990). In addition, *E. plathypenae* venom affects hemolymph ecdysteroid levels in *T. ni* (Kelly and Cou-

Table 2. Delay in the onset of pupation in *L. dispar* injected with active chromatographic fraction of *M. hebetor* venom extract

Treatment	N	Range (days)*	Mean (day)** (± SEM)	Pupal Weight† (mg) (± SEM)
Control (m)	5	20–22	21.2 ± 0.4	628 ± 75
Control (f)	12	22–24	23.3 ± 0.3	1671 ± 122
Fraction (m)	3	24–27	26.0 ± 0.6	597 ± 82
Fraction (f)	12	24–29	26.8 ± 0.6	1957 ± 131

Day 1, third-instar larvae were injected with 1.5 μl saline or 1.5 μl of active chromatographic fraction, 5×10^{-4} μg fraction protein per mg larval weight.

* Days following injection when larvae pupated.

** Mean of all days on which pupation occurred.

† Mean weight on day of pupation.

dron 1990). In preliminary experiments, we found that *M. hebetor* venom gland extract inhibits the production of ecdysteroids by *L. dispar* larvae caused by *M. hebetor* venom is in any way related to a potential effect of the venom on ecdysteroid metabolism.

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