The Biological Effects of Venom Derived from the Ectoparasitic Wasp *Eulophus pennicornis* (Nees) (Hymenoptera: Eulophidae): Evidence for Dual Endocrine Regulation

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Abstract: The venom of the ectoparasitoid *Eulophus pennicornis* is known to induce developmental arrest in parasitized hosts through the prevention of moulting. However, little work has explored how the venom affects different host stages, whether the observed effects vary with different doses, and whether the venom has activity in non-host species.

Injections of venom doses of ≥0.5 μg protein prevented the development of >90% of both fifth and sixth instar *Lacanobia oleracea* larvae. In fifth instars, developmental arrest at doses of ≥ 1.0 μg occurred prior to ecdysis to the sixth stadium, whilst the 0.5 and 0.25 μg treatments allowed 36% and 77% of injected larvae, respectively, to moult although most subsequently failed to pupate. Although naturally parasitized hosts always showed reduced growth, lower doses of venom often prevented pupation without affecting this parameter, particularly in sixth instar larvae where only the highest dose (2.0 μg) reduced the mean maximum weights achieved. Two factitious lepidopteran hosts, *Manduca sexta* and *Spodoptera littoralis*, were also affected by injection of the venom at w/w doses equivalent to those used for *L. oleracea* although larval-larval moulting was only prevented in the former.

Fractionation of venom using gel-filtration FPLC indicated the presence of at least two factors that markedly affected host development. One series of contiguous fractions, on injection, prevented larval-larval ecdysis in a similar way to whole venom. A second series of fractions had no effect on larval-larval ecdysis but resulted in the formation of deformed pupae, an effect that closely resembled the application of a juvenile hormone (JH) analogue, which may indicate the presence of a factor within the venom that modulates host levels of this hormone.

Keywords: *Lacanobia oleracea*, Noctuidae, juvenile hormone, ecdysteroids, parasitoid-host interactions.

INTRODUCTION

Parasitic wasps frequently alter their host's physiology following parasitism through a process known as host regulation [1]. These manipulations are mediated by factors introduced into hosts by the female wasp, such as venom and polydnavirus, larval secretions, or teratocytes derived from the serosal membrane of the parasitoid's egg [1-3]. These factors modify the physiology and immunology of the host in several ways in order to create a resource that is favourable for the development of the wasp's progeny. Such maternally-derived compounds play a crucial role in the reproductive success of many parasitoids and successful development of a parasitoid's offspring frequently cannot proceed in their absence.

Recently, it has been reported that the gregarious ectoparasitoid *Eulophus pennicornis* (Nees) manipulates juvenile hormone (JH) levels in larvae of its host, the tomato moth *Lacanobia oleracea* (L.) [4]. This manipulation leads to a ca. 100-fold increase in JH levels in sixth instars within five days of parasitism. Similar manipulations of host JH levels have been widely reported for a range of parasitoid species [5-7] and up-regulation of this hormone appears to a very common aspect of parasitoid-induced host regulation. Increases in host JH levels are thought, amongst other things, to prevent the well characterized physiological changes associated with pupal commitment that would occur when insects are parasitized close the end of their larval development, especially during the final larval stadium [4,8,9].

Previous investigations using *E. pennicornis* have reported that ecdysteroid titres are markedly altered following parasitism by this parasitoid [10,11], with the magnitude of the manipulation being highly dependent on the larval stadium of the host at the point of parasitism. Manipulation of ecdysteroid titres manifests itself in both parasitized and artificially envenomated hosts as a significant reduction and delay in the increase in 20-hydroxyecdysone (20E) levels that would normally occur prior to ecdysis in non-parasitized hosts [12]. This effect of the wasp's venom was initially thought to be closely allied to the elimination of ecdysis that parasitized or venom-injected caterpillars always exhibit although recent work has indicated that 20E manipulation alone cannot explain the elimination of moulting [8]. A similar situation occurs in the ectoparasitoid *Euplectrus plathypenae* Howard where there is evidence that arrestment factors directly affect the epidermal tissue without the need for interaction with other areas of the body [13]. However, it has been shown in a related species, *Euplectrus comstockii*

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Howard, that different arrestant mechanisms may be operating in different host instars, with 20E levels being significantly affected in *Trichoplusia ni* (Hübner) larvae in the final stadium but not in the preceding larval stage. Such a variable strategy to deal with hosts within different stadia has been similarly indicated for E. pennicornis [4].

Whilst the existing body of work on *E. pennicornis* has shed light on some of the physiological manipulations elicited by this wasp, there has been little exploration of whether the elimination of moulting and upregulation of JH are mediated by a single venom factor or from the combined activity of several different components. Furthermore, the observable effects of venom factors on various aspects of host development have not been reported in any detail, particularly with respect to injection into different host stages, and the response of larvae to different doses. These points are of particular relevance as *E. pennicornis* has the ability to parasitize a range of stages of *L. oleracea* (fourth, fifth and sixth stadium larvae) and to vary its clutch precisely in proportion to the size of the host [14]. Furthermore, the reproductive success of this parasitoid appears linked to both its ability to identify the highest quality hosts and to successfully regulate their development [8,14]. These observations are best illustrated by the fact that late-parasitized hosts (i.e. those within 3-4 days of pupation) continue to develop following parasitism, and form prepupae, a developmental event that leads to very poor parasitoid larval survival; whereas larvae parasitized at the start of the final stadium prove to be optimal and are preferentially parasitized by this wasp [14].

The developmental arrest that always occurs in hosts following parasitization by *E. pennicornis*, or artificial envenomation [15], may mean that other, potentially observable, venom-induced effects are masked. In the present study, experiments were designed to investigate the effect of a range of venom doses on the host, so that the full potential spectrum of host responses might be observed. The developmental effects of the venom were also explored in two factitious hosts that *E. pennicornis* does not parasitize. Following this, through partial separation of the factors present in the wasp's venom, it was also investigated as to whether different types of activity (i.e. distinct effects on the host) could be observed in *L. oleracea* larvae that mirror the various effects induced through injection of varying doses of whole venom. In order to equate some of the observed developmental outcomes with JH manipulation, the effects of some venom fractions were compared with the morphogenetic disruption caused by the exogenous application to host larvae of the JH analogue methoprene.

MATERIALS AND METHODS

Insects

Newly emerged *E. pennicornis* adults were derived from a laboratory culture maintained on late stadium larvae of the tomato moth, *L. oleracea*, at 25°C, 70% R.H. and 16h:8h L:D according to the methods described previously [16]. *Lacanobia oleracea* larvae were derived from a laboratory culture maintained at 20°C and 16h:8h L:D and reared on artificial diet [17] in plastic sandwich boxes (150x150x75 mm) covered with muslin lids. Procedures for rearing, staging, and synchronising larvae have been described previously [18]. Experiments utilized larvae in the fifth and sixth stadia only. All experiments were carried out at 25°C, 70% R.H. and 16h:8h L:D.

Larvae of the cotton leafworm, *Spodoptera littoralis* (Boisduval), were derived from a laboratory culture and reared following essentially the same procedures as described for *L. oleracea*. Larvae of the tobacco hornworm, *Manduca sexta* (L.), were reared from eggs kindly supplied by Prof. Stuart Reynolds (University of Bath) and reared as previously described [19].

Preparation of *E. pennicornis* Venom

Mixed age female wasps (approx. 1-2 weeks old) were removed from culture and chilled at 4°C for approximately 30 min. The wasps were then immersed in a vial of phosphate buffered saline dissection buffer (PBS, 25 mM NaH_2PO_4, 25 mM $NaHPO_4$, 155 mM NaCl, 7% sucrose) and held over ice for a further 10 minutes. Wasps were subsequently transferred to the stage of a dissecting microscope and placed in a small volume (ca. 50 µl) of chilled dissection buffer. The venom sacs of the wasps were removed through gently pulling on the ovipositor with a pair of fine forceps to reveal the sac. The removed sacs were then placed in a 1.5 ml microcentrifuge tube held over ice until approximately 50-100 sacs had been collected. The venom sacs were gently macerated and debris removed through centrifugation at 10,000 g, 4°C, for 10 min. The venom preparations were stored at –20°C until use.

Activity of Crude Venom

The anti-moulting activity of crude venom was investigated against *L. oleracea* larvae. The protein content of venom preparations was determined using the method of Bradford [20] using BSA as a standard. Dilutions of the venom were prepared to produce doses that would deliver 0.05 µg to 2 µg protein content when injected into hosts in 5 µl of dissection buffer.

Newly moulted fifth or sixth instar *L. oleracea* larvae (< 12 h after ecdysis) were removed from culture and chilled over ice. Subsequently, the insects were anaesthetized with CO_2 and injected with 5 µl of a crude venom dilution using a 10 µl Hamilton syringe. The larvae were place individually in 250 ml plastic pots, provided with artificial diet *ad libitum* and monitored until death or pupation. Developmental events such as moulting, the occurrence of supernumerary stadia, and pupal deformity were recorded and 10-33 larvae were injected for each instar and dose. A sample of injected fifth instar *L. oleracea* (n = 10-15 for each dose) and all (n = 10-20) sixth instar larvae were also weighed daily. As well as the usual laboratory host of *E. pennicornis*, injections were also performed using two factitious hosts. In the case of *S. littoralis*, insects were selected from laboratory culture as newly ecdysed fifth instar larvae, injected as described above using the 0.5µg dose, and growth and development monitored until death or pupation. Larvae of *M. Sexta* were raised individually up to the start of the fourth stadium, injected with 5 µg of venom protein (in 10 µl of dissection buffer) and subsequently maintained at 20°C, 60% R.H, 16:8 LL:DD. Controls for both species were injected with equivalent volumes of buffer only.

Fractionation of *E. pennicornis* Venom

Venom samples were subjected to separation using a fast pressure liquid chromatography (FPLC) system fitted with a Superose-12 HR 10/30 gel filtration column coupled to a Beckman 168 diode array detector. After equilibrating the column with Dulbecco's PBS (Sigma), fractionation was initiated by injecting ~1000 µg of venom protein (ca. 300 venom sacs) in 0.2 ml of dissection buffer onto the column. Proteins were eluted with buffer at a flow rate of 0.4 ml/min. The UV detector was set at 280 nm and monitored at 0.4 AUFS sensitivity using Beckman 32 Karat software. Fractions (0.4 ml) were collected over a period of 75 min at 1 min intervals. The bed volume of the column was 24 ml and the void volume 7.6 ml. Bio-Rad protein standards for gel filtration were used for column calibration.

Venom fractions from several gel filtrations were pooled in batches of three consecutive fractions. Subsequently, these pooled fractions were concentrated by ultrafiltration using Millipore Ultra-free-MC centrifugal concentrators (5,000 NMWL) spun at 5000g 4°C until volumes had been reduced by 50-60% (typically 1-2 hours). These pooled fractions were bioassayed for activity (see below) and, on the basis of the results obtained, individual 1 min fractions were collected from further FPC separations using the procedures described above, concentrated and subjected to further investigation.

Biological Activity of *E. pennicornis* Venom Fractions

The biological activity of FPLC fractions was examined through bioassay against fifth instar *L. oleracea*, as described above for whole venom. Ten *L. oleracea* were injected with 5 µl of pooled sample of three contiguous fractions. The injected insects were kept individually and monitored until death or pupation. Control insects received 5 µl of Dulbecco's PBS only. A total of 24 pooled fractions were assayed (eluting between 10 to 72 minutes) and any developmental abnormalities were noted.

Where the injection of pooled samples revealed observable activity, further injections using the individual 1 min fractions spanning these areas were undertaken. The protein content of each individual fraction was determined prior to injection (as described above). In addition to noting developmental abnormalities, the effect of selected fractions on the growth of *L. oleracea* was also measured. In these cases, larvae injected at the start of the fifth stadium were weighed daily until pupation or death.

The activity of some fractions appeared to have activity of a type similar to the exogenous application of juvenile hormone to lepidopteran larvae. In order to explore this observation further, a number of newly emerged fifth and sixth stadium *L. oleracea* were topically treated with the juvenile hormone analogue (JHA) (S)-methoprene. Solutions of 50 µg μl^{-1} and 100 µg μl^{-1} of the JHA were prepared in acetone and cohorts of 15-20 insects, within 12 h of moulting to the fifth or sixth stadium, were treated with 1.0 µl of one of the two concentrations. Control insects were treated with 1.0 µl of acetone only. Subsequently, all insects were monitored for developmental normality, as described above. A proportion of the insects were weighed daily to allow comparisons to be made with larvae injected with venom or venom fractions. Totals of 10-20 insects of each instar were treated with each methoprene dose.

Statistical Analysis

Statistical analysis was conducted using StatsDirect 2.6.1. The calculation of LD_{50} values for crude venom was achieved using Probit analysis. Effects on host weights and developmental times were analyzed using one-way analysis of variance (ANOVA) and, where appropriate, means separated by Dunnet multiple range *post-hoc* tests for the comparison of treated insects with the controls. Unpaired Student's t-tests were used for the comparison of two means. In all cases the accepted level of significance was $P < 0.05$.

RESULTS

Activity of Crude Venom

The effect of crude venom preparations on the normal development of fifth and sixth instar *L. oleracea* is shown in Fig. (**1**). Venom doses of 0.1 µg and above had a marked effect on the successful development of larvae injected at the start of the sixth stadium. As a result, only 25% of those insects injected with 0.1µg of venom protein (0.6 µg g^{-1}) at the start of the final larval stadium developed normally and formed viable pupae whilst doses of 1.0 µg and above eliminated all normal development. Fifth instar larvae were less affected by the 0.1µg venom dose (1.6 µg g^{-1} fresh weight) than sixth instar caterpillars, with over 60% developing normally whilst the effect of higher doses was essentially the same as that of the final stadium larvae. When the dose-responses for both larval stages were examined, approximate LD_{50} values of 2.8 µg g^{-1} and 0.58 µg g^{-1} were calculated for fifth and sixth instar larvae, respectively.

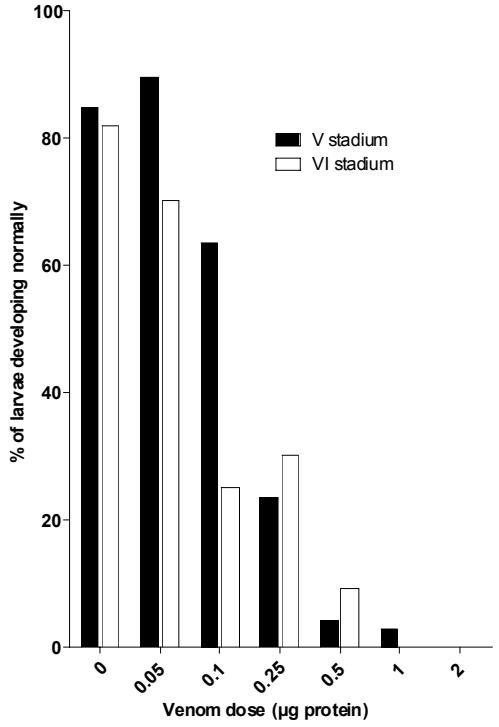

Fig. (1). The proportion of venom injected fifth and sixth stadium *L. oleracea* larvae that developed normally to form viable pupae following injection with *E. pennicornis* venom at the stated dose.

Table 1. The Effect on the Successful Development of *L. oleracea* Larvae Following Injection with Doses of Crude Venom at the Start of Either the Fifth or Sixth Stadium

Venom Dose (µg Protein)	Approx. Dose (µg / g Insect)	N Injected	Moulted to L6 (%)	Mean Period Spent in Fifth Stadium (days)	Mean Time to Pupation / Death (days)	Supernumerary Stadia (%)	Pupated (%)	Deformed Pupae (%)(N Deformed / N Pupae)
Fifth instars								
0.0	0.0	32	100.0	3.9 ± 0.10	11.5 ± 0.45	0.0	90.6	6.3 (2/29)
0.05	0.8	28	100.0	4.3 ± 0.34 (ns)	12.1 ± 0.34 (ns)	0	100.0	10.7 (3/28)
0.1	1.6	30	93.3	4.6 ± 0.24 (ns)	12.0 ± 0.40 (ns)	10.0	86.7	26.9 (7/26)
0.25	4.0	30	76.7	4.8 ± 0.26 (ns)	12.1 ± 0.56 (ns)	20.0	40.0	41.7 (5/12)
0.5	8.0	25	36.0	7.4 ± 0.50 (***)	9.4 ± 0.53 (*)	4.0	8.0	50.0 (1/2)
1.0	16.0	35	14.3	8.4 ± 0.56 (***)	9.3 ± 0.58 (*)	0.0	2.8	0.0 (0/1)
2.0	32.0	25	0.0	9.3 ± 0.80 (***)	9.3 ± 0.80 (*)	0.0	0.0	0.0 (0/0)
Sixth instars								
0.0	0.0	11	-	-	8.0 ± 0.54	0.0	90.9	10.0 (1/10)
0.05	0.3	10	-	-	6.1 ± 0.78 (ns)	0.0	80.0	12.5 (1/8)
0.1	0.6	20	-	-	6.0 ± 0.74 (ns)	0.0	65.0	69.2 (9/13)
0.25	1.5	20	-	-	7.9 ± 1.25 (ns)	20.0	55.0	45.5 (5/11)
0.5	3.0	11	-	-	7.5 ± 0.73 (ns)	0.0	9.1	66.6 (2/3)
1.0	6.0	10	-	-	9.0 ± 0.91 (ns)	0.0	20.0	100.0 (2/2)
2.0	12.0	10	-	-	9.2 ± 1.21 (ns)	0.0	0.0	0.0 (0/0)

The developmental times of hosts were analysed by one-way ANOVA and the means of venom-injected hosts compared with the control means using Dunnet multiple comparison tests. The level of significance is indicated as ns = $P > 0.05$, * $P < 0.05$, *** $P < 0.001$.

In the fifth instar hosts there was a progressive increase in the number of larvae that failed to moult as venom dose was increased although only the highest dose eliminated ecdysis (Table **1**). However, successful ecdysis did not always lead to the successful completion of development (i.e. normal pupation). This fact was best illustrated by larvae injected by the 0.25 µg dose where 76.7% moulted to the sixth stadium but only about 20% went on to form viable pupae, the remainder becoming moribund or producing deformed pupae. Sixth instar larvae demonstrated a similar dose response to the increasing venom doses as fifth stadium larvae with caterpillars treated with the highest dose (2.0 µg) all becoming arrested prior to pupation whilst only a small proportion of insects treated with the 0.5 and 1.0 µg doses formed pupae, of which most were deformed.

The formation of deformed pupae was most evident in sixth instar larvae, particularly those injected with the 0.1 µg dose where nine out of the 13 pupae (69%) produced were non-viable (Table **1**). Injection of venom into fifth instar caterpillars significantly increased the period of time spent within that stadium ($F_{6,198} = 27.27$, $P < 0.001$) from 3.9 d in control larvae to 9.3 d in insects injected with 2.0 µg venom protein. However, the duration of the sixth stadium, either to pupation or death, was not extended significantly in caterpillars injected at the start of that stage ($F_{6,63} = 2.01$, $P = 0.078$) (Table **1**).

In the case of fifth instars, a venom dose of ≥0.5 µg of protein resulted in the near total elimination of growth similar to that seen in parasitized hosts (Fig. **2A**). When the mean maximum weights that caterpillars achieved post treatment were examined, it was apparent that doses of ≥ 0.5 µg had a significantly deleterious effect on their capacity to grow (one-way ANOVA, $F_{6,82} = 39.21$, $P < 0.001$) (Fig. **2B**). However, it was notable that the maximum weights achieved by larvae injected with the 0.1 and 0.25 µg venom doses did not differ significantly from the controls (Dunnet multiple comparisons, $P > 0.05$) despite the fact that both treatments reduced developmental success. Venom injection had a significant, although comparatively reduced effect, on sixth instar larval growth that was highly dependent on the dose delivered (one-way ANOVA, $F_{6,79} = 3.23$, $P < 0.01$) (Fig. **2C**,**D**). Only the 2.0 µg dose consistently reduced growth to the extent that the maximum weight achieved by treated larvae was significantly less than that for controls (Dunnet multiple comparisons, $P < 0.01$) (Fig. **2D**), despite doses of 0.1 µg and above markedly affecting developmental success and causing slower rates of growth (growth curves not shown).

The injection of *E. pennicornis* venom had a profound effect on the survival of two factitious hosts (Table **2**). When injected with a protein dose (0.5 µg) that consistently resulted in developmental arrest in most *L. oleracea*, the majority of fifth instar *S. littoralis* similarly became developmentally arrested (80%). This cessation in development occurred at a point just prior to when ecdysis would normally have occurred in the majority (70%) of the treated larvae (Table **2**). As a result, the maximum weight achieved by

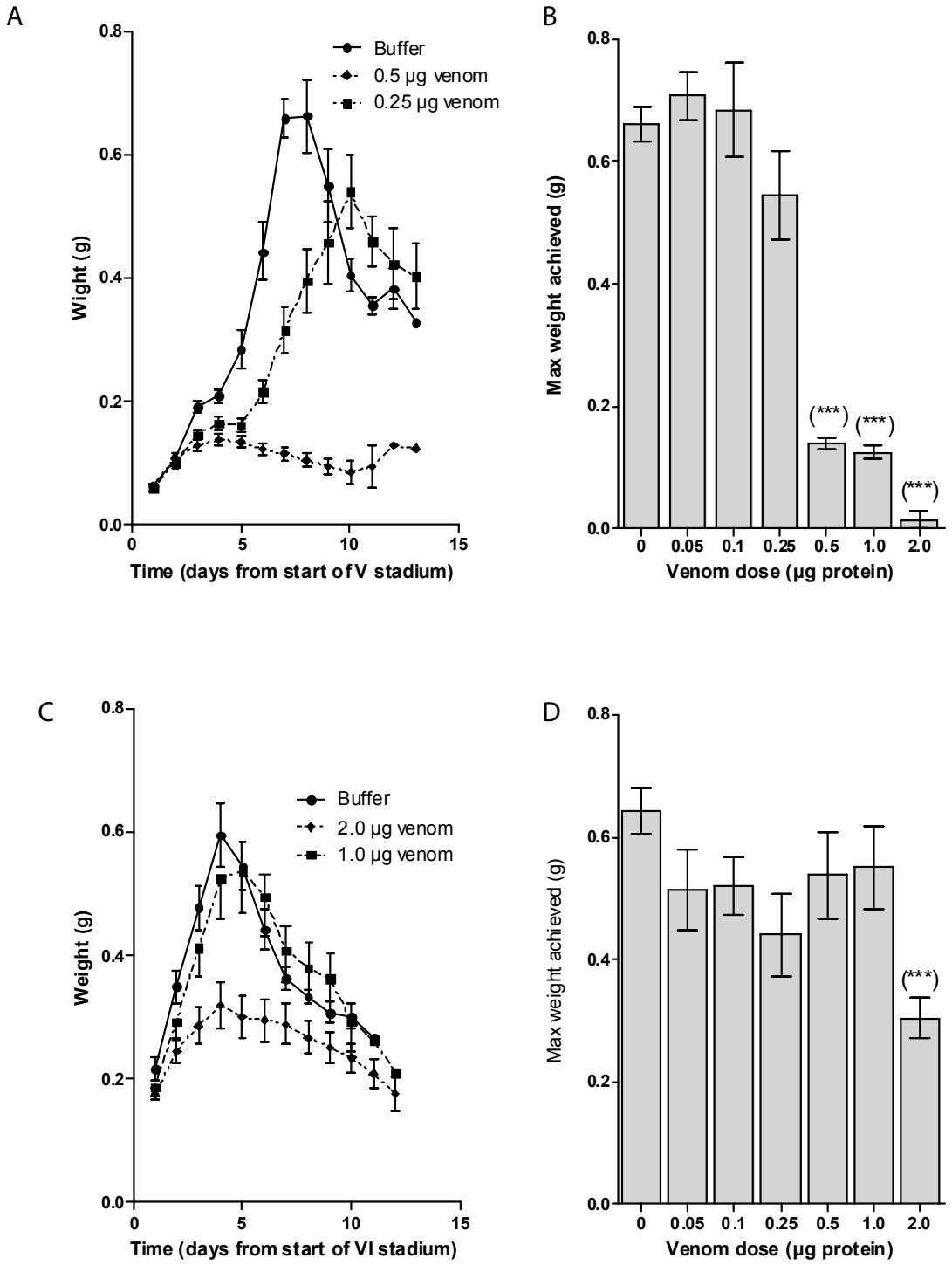

Fig. (2). The effect of *E. pennicornis* venom on the growth of *L. oleracea* larvae injected at the beginning of the fifth and sixth stadium. (**A**) Growth curves of fifth instar larvae at selected doses and (**B**) maximum weights attained for all doses. (**C**) The growth of sixth instar larvae at selected doses alongside (**D**) the maximum weights achieved by larvae for all doses.

venom-injected larvae, at 0.20 ± 0.07g, was significantly less than the 0.84 ± 0.04 g attained by the control insects ($t = 7.75$, $df = 18$, $P < 0.001$). *Manduca sexta* larvae injected with 5 µg of venom protein at the start of the penultimate (fourth) stadium, whilst retaining the capacity to moult, all failed to pupate and became moribund during their final larval stadium ca. 20 days after injection (Table **2**). Following ecdysis to the fifth stadium, all larvae continued to grow at a reduced rate for up to two weeks but only achieved a maximum weight of 4.46 ± 0.94 g, a value significantly less than the control average of 8.86 ± 0.27 g ($t = 5.13$, $df = 18$, $P < 0.001$) (Fig. **3A**, **B**). All larvae turned black at a point just

Table 2. The Effect on the Successful Development of *S. littoralis* and *M. sexta* Larvae Following Injection with Doses of Crude Venom at the Start the Penultimate Larval Stadium

Venom Dose (µg Protein)	Approx. Dose (µg / g Insect	N injected	Moulted to Final Larval Stadium (%)	Supernumerary Stadia (%)	Pupated (%)	Deformed Pupae (%) (N Deformed/N Pupae)
S. littoralis (L5)						
0.0	0.0	10	100.0	0.0	100.0	10.0 (1/10)
0.5	8.0	10	30.0	0.0	20.0	0.0 (0/2)
M. sexta (L4)						
0.0	0.0	10	100.0	0.0	90.0	0.0 (0/9)
5.0	15.0	10	100.0	0.0	0.0	0.0 (0/0)

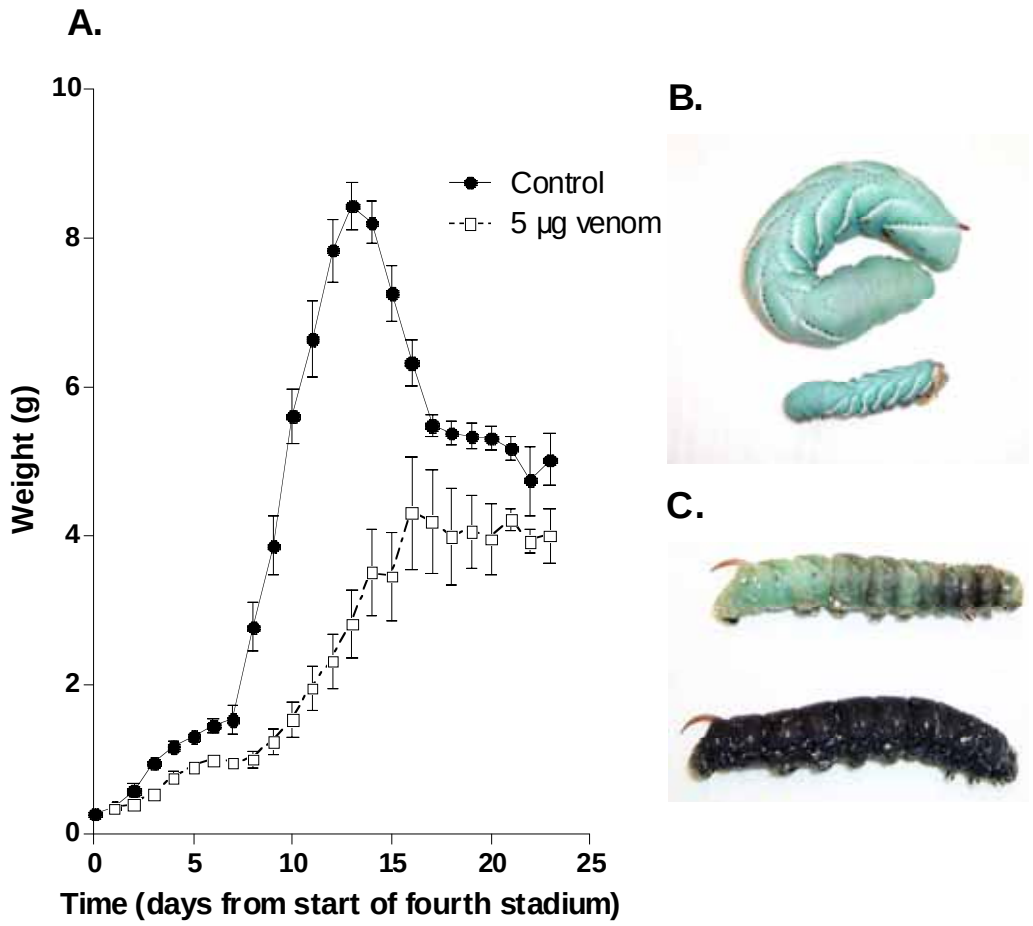

Fig. (3). (**A**) The effect of the injection of 5.0 µg of venom into fourth instar *M. sexta* larvae. (**B**) The comparative sizes of control (top) and injected hosts (bottom) 13 days after treatment and (**C**) the melanization that occurs in treated larvae prior to death (ca. 20 days post treatment).

prior to the point when the normal onset of pupation would occur and died one or two days later (Fig. **3C**).

Activity of FPLC Fractions and Comparisons with the Juvenilizing Activity of Methoprene

A preliminary screen of FPLC fractions indicated the presence of two series of fractions that contained significant activity and resulted in developmental dysfunction in injected insects (results not shown). These fractions were subjected to further investigation following concentration to approximately 40% of their initial volume. Fractions 26-33 resulted in moulting from the fifth to the sixth stadium being eliminated in some or all insects. This data indicated the presence of an anti-moulting factor (AMF) although the fact that most arrested insects died after commencing ecdysis (Fig. **4B**) meant that the effect differed somewhat from naturally stung hosts where most insects do not show any attempt to moult. The injection of the most active fractions (26-30) gave largely similar levels of anti-moulting activity with the exception of fraction 30 where 50% moulted and went on to

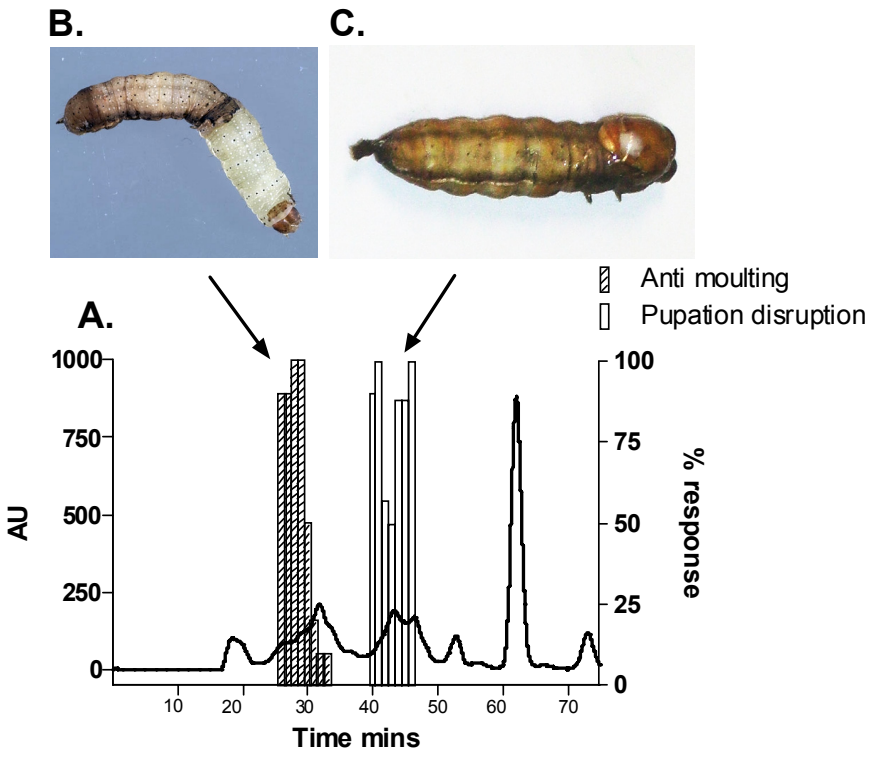

Fig. (4). (**A**) The elution of the anti-moulting and JH-modulating-like factors (see text for details) and the percentage of injected larvae (% response) exhibiting the pathologies associated with (**B**) the anti-moulting factor (AMF) and the (**C**) putative JH modulating factor (pJHMF).

Table 3. The Effect of Concentrated FPLC Fractions on the Development of *L. oleracea* Larvae Injected at the Beginning of the Fifth Stadium and Comparisons with the Developmental Effects of Topical Applications of the JHA Methoprene to Fifth and Sixth Instar Larvae

Activity Type / Fraction Number	N Treated	Total Protein Injected per Insect (µg)	Successful L5-L6 Moult (%)	Pupation (%)	% Deformed Pupae (N Deformed / N Pupae)
Moult inhibitory fractions (AMF) L5					
Buffer (PBS)	20	0.0	90.0	80.0	6.3 (1/16)
26	10	0.37	10.0	10.0	100.0 (1/1)
27	10	0.33	10.0	10.0	0.0 (0/1)
28	10	0.41	0.0	0.0	0.0 (0/0)
29	10	0.62	0.0	0.0	0.0 (0/0)
30	10	0.48	90.0	50.0	100.0 (5/5)
JH-like activity (pJHMF) L5					
43	10	0.16	100.0	80.0	87.5 (7/8)
44	10	0.05	100.0	90.0	74.8 (7/9)
45	10	0.09	100.0	90.0	88.9 (8/9)
46	10	0.06	90.0	80.0	100.0 (8/8)

(Table 3). Contd…..

Activity Type / Fraction Number	N Treated	Total Protein Injected per Insect (µg)	Successful L5-L6 Moult (%)	Pupation (%)	% Deformed Pupae (N Deformed / N Pupae)
Methoprene applications L5					
0.0	20	-	85.7	81.0	0.0 (0/17)
50.0	20	-	100.0	90.0	38.9 (7/18)
100.0	20	-	100.0	85.0	52.9 (9/17)
Methoprene applications L6					
0.0	20	-	-	100.0	5.0 (1/20)
50.0	20	-	-	60.0	90.9 (10/11)
100.0	20	-	-	20.0	100.0 (4/4)

form deformed pupae (Table **3**). Fraction 27 was seen to be the most active with a protein dose of 0.33 µg completely eliminating ecdysis. However, whilst almost all other fractions allowed for the completely normal development of >85% of injected caterpillars (results not shown), the formation of viable pupae was markedly inhibited by a small number of samples (fractions 40-46). In these fractions the majority of injected larvae moulted normally to the sixth instar, continued to feed, and developed normally up to the point of pupation whereupon they either failed to pupate or formed a non-viable deformed pupa (Fig. **4C**). In the case of fraction 43, 60% of those initially injected produced deformed pupae whilst the remainder died just prior to pupation, often at the point where head capsule slippage had occurred. In most cases, pupation (attempted or complete) of insects injected with fractions 43-46 took place 2-3 days after the control insects (data not shown). Activity of this type closely resembled exogenous juvenile hormone (JH) treatment and these fractions were considered as potentially containing a factor that affects JH levels (putative JH modulating factor, pJHMF).

The growth of insects injected with the AMF fractions closely resembled that of insects injected with whole venom (Fig. **5A**) such that of larvae at the start of both the fifth and sixth stadium showed markedly reduced growth followed by developmental arrest. The juvenilizing effects of the pJHMF fractions were compared with topical applications of the JHA methoprene. Injection of the pJHMF (using a pooled sample of fractions 40-46) allowed for the continued growth of most larvae for several days longer than that of control insects (Fig. **5A**) followed by the formation of deformed pupae. This effect closely resembled that of the topical application of 100 µg methoprene, particularly when the JHA was applied at the start of the sixth stadium (Fig. **5B**) where growth rate was initially similar to the control insects. The effect of application of methoprene at the start of the fifth

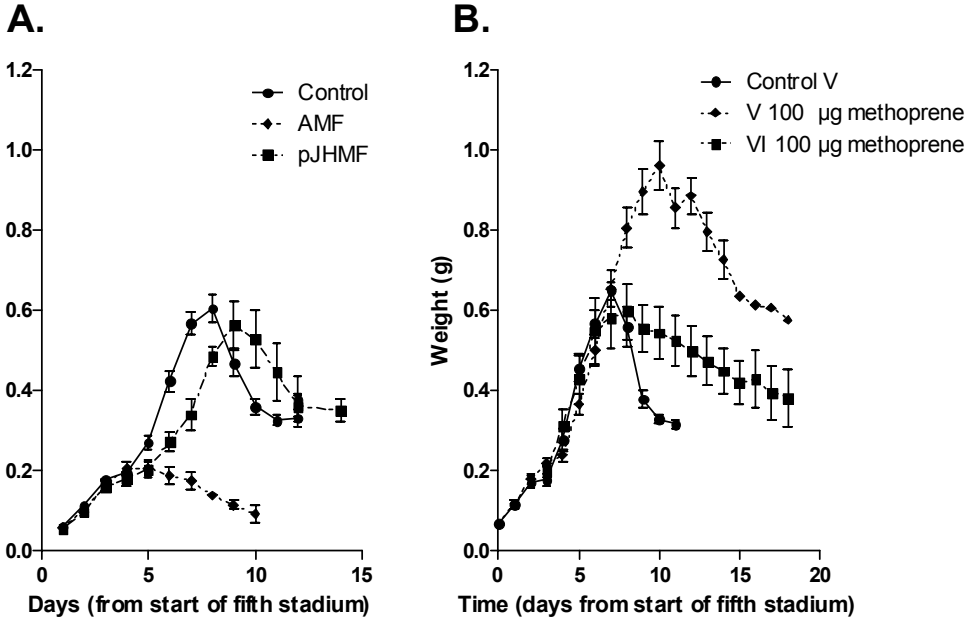

Fig. (5). The effect on growth of the AMF and pJHMF factors when injected into fifth instar *L. oleracea* larvae (**A**) and the growth of caterpillars when topically treated with 100 µg of methoprene at the start of either the fifth or sixth stadium (**B**).

stadium, however, was less similar with insects growing much larger during a significantly extended sixth stadium of approximately 14 d (control duration ca. 7 d) ($t = 11.19$, $df = 36$, $P < 0.001$). Despite this considerable extension of the final larval stadium, and the larvae attaining much larger weights, 85% of fifth instar larvae treated with methoprene were able to form pupae (viable and non viable) as opposed to 20% in sixth stadium larvae treated with the equivalent JHA dose.

DISCUSSION

The crude venom of *E. pennicornis* is highly active and induces developmental arrest at relatively low levels of injected protein. The pathology observed is similar to that previously reported [10,15,16] with insects ceasing to feed 2-4 d after injection, becoming moribund and failing to moult to the next larval stadium. The injection of doses of venom at levels below those that reliably prevent ecdysis revealed that prevention of successful pupation frequently occurs even if the last larval-larval moult is not prevented. A range of other developmental abnormalities, such as the occurrence of supernumerary stadia and incomplete ecdysis, also occur at low venom doses, pathologies that were not reported in earlier work on the activity of the venom of this wasp. The induction of supernumerary stadia, in particular, is indicative of the JH manipulation that is known to occur in parasitized hosts [4], a phenomenon that is usually masked by the anti-moulting activity of the venom. However, the marked growth reduction that is characteristic of natural envenomation was only associated with the AMF in injected *L. oleracea* and insects injected with the pJHMF grow more or less normally. Notably, in terms of the venom dose per unit fresh weight required to prevent the successful completion of larval development, sixth stadium larvae appear much more susceptible to the developmental perturbations induced by the venom than the much smaller fifth instar larvae.

Whilst the two distinct biological effects could be construed as being brought about by the diminishing effects of a single factor, the fractionation of venom by FPLC effectively separated the two types on activity, producing fractions that predominantly, though not exclusively, gave either anti-moulting or JH-like activity. The first phenomenon, which has been widely reported as an effect of *E. pennicornis*'s venom, is crucial to the survival of larvae developing on fifth (penultimate instar) hosts that would be shed with the cast cuticle upon ecdysis. The second effect has not been reported upon previously and may be caused by the factor responsible of the increased levels of juvenile hormone that are observed in hosts parasitized at the start of the final larval stadium [4]. The malformed pupae observed following the injection of certain fractions are highly reminiscent of larvae that have been exposed to abnormally high levels of JH during their final instar, a time when JH is virtually absent in larval Lepidoptera [12,21]. It is, therefore, likely that this second biological activity results from a JH-modulating factor that targets either JH biosynthesis or its metabolism by juvenile hormone esterase, both effects that have been recorded from *L. oleracea* parasitized by *E. pennicornis* [4] and a number of other parasitoid-host associations [6,22]. The current work also indicates that the JH-like activity would appear to only come into effect in final (sixth) instar larvae, even when caterpillars are injected (or envenomated) in the preceding stadium. This supposition is substantiated by the fact that topical applications of methoprene onto fifth stadium larvae produced much more marked effects on growth that when it is applied to sixth instar caterpillars, where the observed effects very closely resembled injection of the pJHMF into penultimate instar larvae that subsequently go on to moult to this stadium. Such observations provide further evidence to substantiate the fact that *E. pennicornis*, and other parasitoids, use maternally-derived parasitoid factors to manipulate different host stages in different ways [4,8, 23,24].

Whilst good separations of biological activity were achieved by gel-filtration, the fractions remained complex and, following additional concentration, in excess of 20 protein bands were still present in the AMF samples and ca. 5 in the pJHMF fractions when separated by polyacrylamide gel electrophoresis and stained with Coomassie blue (results not shown). Work is ongoing to purify the venom fractions further so that accurate characterization and partial sequencing of the physiologically active factors present in *E. pennicornis* venom can be achieved. Furthermore, measurements of 20E and JH following injection of the AMF and pJHMF are strongly indicated to confirm the biological inferences that are made here.

The current findings provide further evidence for the dual endocrine regulation strategy employed by *E. pennicornis* that has been alluded to previously, and provides a correlation between existing endocrinological measurements and biological observations [4]. In whole venom, the anti-moulting activity masks other underlying activities of which, as is shown here, includes a factor(s) that induces JH-like effects and probably operates at comparatively lower concentrations than the AMF. It was noted, however, that some fractions containing the AMF also induce some deformity in pupae that derive from hosts that were not prevented from moulting, an observation that requires further study to determine if the deformity differs qualitatively from that induced by the pJHMF. It is also important to note that the anti-ecdysis activity of the AMF fractions did not exactly reflect that seen in naturally envenomated hosts, or those injected with whole venom, in that partial ecdysis almost always occurs. This could be construed as a response to a sub-optimal dose of the AMF or, potentially, could be due to the fact that more than one factor is responsible for the complete elimination of all moulting activity.

Previous work [8,14] has shown that early sixth stadium hosts (0 - 48 h post ecdysis) are preferentially parasitized by *E. pennicornis* and suggest that juvenilization, and the capacity to induce it, have been selective drivers in this parasitoid preferentially parasitizing hosts of this age. The powerful juvenilizing effect that a component of *E. pennicornis*'s venom induces in the host lends credence to the fact that it is probably critical for the wasp to ensure that hosts do not become physiologically committed to pupation once they have been parasitized. This need is likely to be necessitated by the specific nutritional needs of the parasitoid's larva, requirements that are not met by insects that are physiologically committed to the production of pupal tissues through the absence of JH that typically occurs during the ultimate larval instar of *L. oleracea* and other lepidopterous larvae. As JH manipulation to prevent pupal commitment is only required for the regulation of sixth instar larvae, it is therefore likely

that *E. pennicornis* has developed strategies for the manipulation of hosts that only come into effect when hosts enter this stadium.

It was also interesting to observe that whilst whole venom induces effects in the closely related factitious host *S. littoralis* indistinguishable to those seen in *L. oleracea*, in that moulting is severely disrupted, only the JH-like effects manifest themselves in the more distantly related *M. sexta* at a comparable weight-for-weigh dose. However, it was noted that growth is markedly reduced in *M. sexta* injected with whole venom, despite the fact they are unaffected by the anti-moulting factor contained within it. This contrasts with findings from *L. oleracea* where growth modulation is always associated with anti-moulting activity. Although the activity of some parasitoid venoms have been shown to operate well beyond their natural host range [25], the partition of activity observed in *M. sexta* is extremely interesting and may provide a very useful tool for the further study of the biologically active components of *E. pennicornis*'s venom at the physiological and biological level.

Much recent work into the host regulatory effects of parasitoid venoms has been driven by the need to find biologically active compounds that may be used as novel insecticides [26]. In the case of *E. pennicornis*, at least two distinct anti-insect factors are detected within the venom with biological activities that have the potential to be highly specific to lepidopteran larvae. Work is currently ongoing to further characterize these and other factors present within the venom of this parasitoid with a view to exploiting their anti-insect potential in the future.

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