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Effects of Envenomation on Survival and Development of the Host-Parasitoid System of *Galleria mellonella* (Lepidoptera: Pyralidae) and *Bracon hebetor* (Hymenoptera: Braconidae)

Ferzana Bhatti[1], Fiaz Hussain[2], Zain ul Abdin[1]*, Muhammad Arshad[1], Saqi Kosar Abbas[2] and Muhammad Zeeshan Shabbir[3]

[1]*Department of Entomology, University of Agriculture, Faisalabad-38040, Pakistan*
[2]*College of Agriculture, BZU Sub Campus Bahadur, Layyah-31200, Pakistan*
[3]*State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China*

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FB performed the experiment. FH assisted in experiments and the analysis of data. ZA designed and supervised the experiment and improved the manuscript. MA reviewed the article and made improvements. SKA contributed to the analysis of data and interpretation of results. MZS contributed to the writing of the article.

Key words
Ectoparasitoid, Venom injection, Dufour's gland, *G. mellonella*, Mortality

ABSTRACT

The role of venom injection by an ectoparasitoid, *Bracon hebetor* (Say) (Hymenoptera: Braconidae) in inducing paralysis and developmental arrest in its host, *Galleria mellonella* (L.) (Pyralidae: Lepidoptera) was studied. Bioassays were performed with microinjections carrying crude and diluted venom and secretion of Dufour's gland of *B. hebetor* into healthy and mature host larvae. To observe the effects of venom injection on development of parasitoid progeny, freshly laid eggs of the parasitoid were transferred from stung hosts onto non-stung hosts. Venom caused 100% mortality of the host even in diluted forms; Dufour's gland secretion did not cause any significant mortality. The secretions injected by an ovipositing female not only contributed to a reduction in egg mortality but also positively affected survival and development of immature insect parasitoids. These will be helpful to explore novel bioactive molecules useful for sustainable insect biological control programes, especially from the insect parasitoids of Pyralidae (Lepidoptera).

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INTRODUCTION

The effects of parasitic wasps on host physiology, behavior and development are critical components in any host-parasitoid relationship, which result in the mortality of the host in favor of the parasite. Parasitoids, in the order Hymenoptera, exhibit various regulation strategies on their hosts, varying from simple immune repression (Schmidt-Mende *et al*., 2001; Lavine and Strand, 2002; Malva *et al*., 2004; Pennacchio *et al*., 2014; Falabella, 2018) to neuroendocrine ailments related to reproductive and developmental interruptions (Lawrence and Lanzrein, 1993; Pennacchio *et al*., 2001). Host and parasitoid interactions are also exemplified by dramatic parasitoid-mediated host disorders, often linked with paralysis and rapidly leading to host demise. In both parasitic wasp groups, endo- and ecto-parasitoids, the toxic secretions injected by females at the time of egg laying causes the induction of major host physiological alterations following parasitism (Varricchio *et al*., 1999; Moreau and Asgari, 2015). These secretions, which are injected along with the eggs, contain a venom component, which is associated in certain braconid and ichneumonid wasps with a symbiotic virus in the family Polydnaviridae (Asgari, 2006; Pennacchio and Strand, 2006; Gundersen-Rindal *et al*., 2013; Salvia *et al*., 2017, 2018; Quicke and Butcher, 2021). Moreover, host regulation can also be due to secretions from the parasitoid eggs or embryo-associated cells, such as teratocytes, and secretions from the developing parasitoid larvae (Asgari and Rivers, 2011; Salvia *et al*., 2019).

The host regulation phenomena in parasitic wasps is very complex, which causes a sole physical infection (Vinson and Iwantsch, 1981; Digilio *et al*., 2000). Venoms are found in at least six insect orders and accomplish diverse functions including predation, communication, defence against predatory insects, and paralysis of their host for subsequent feeding by their offspring (Schmidt, 1982; Battaglia *et al*., 2014; Laurino *et al*., 2016; Arbuckle,

* Corresponding author: zainunibas@gmail.com
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2017; Schendel *et al*., 2019; Scieuzo *et al*., 2021). The important aspect of this last function, feeding by offspring, is primarily demonstrated by parasitoids for which venom injection and stinging behaviour are essentially related to the parasitoid's reproduction. Venom injection may assist the parasitizing female to oviposit smoothly without any interruption from the host (Cusumano *et al*., 2018). Therefore, the venom has a significant contribution to the positive development of parasitoid progeny. The venom of many parasitoids exhibits virulence effects on their hosts aiding in the egg laying process (Beard, 1978; Stoltz, 1986; Coudron, 1991). Different studies reveal that reproductive secretions. e.g., venom (Wago and Kitano, 1985) and ovarian proteins (Webb and Luckhart, 1994) inserted by parasitizing females can disrupt cytoskeleton of the host hemocytes resulting in reduced cellular immunity (Salvia *et al*., 2021). The ectoparasitoid's venom primarily arrests development of the host through paralysis or by inhibiting host ecdysis (Beard, 1952, 1978); Shaw, 1981; Pike *et al*., 1982; Visser *et al*., 1983; Piek and Spanjer, 1986; Coudron *et al*., 1990; Coudron, 1991; Rivers *et al*.,1993; Rivers and Denlinger, 1994), followed by prevention of the parasitic wasp progeny from being disrupted throughout development.

One of the most widely studied host-parasitoid associations is *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) and *Bracon hebetor* (Say) (Hymenoptera: Braconiodae). *Bracon hebetor* is a larval ectoparasitoid of several lepidopteran pest species (Magro and Parra, 2001; Jhansi and Babu, 2002; Fagundes *et al*., 2005; Yasodha and Natarajan, 2006; Shojaei *et al*., 2006; Desai *et al*., 2007; Kyoung *et al*., 2008; Mohapatra *et al*., 2008). Parasitoid females first hypodermically inject a minor dose of venom in to mature host larvae which causes complete paralysis within 15 min, and then the female deposits 3–20 eggs on the external surface of the host body (Beard, 1952; Benson, 1973; Hagstrum and Smittle, 1977).

Galleria mellonella is a very suitable host for the mass rearing of *B. hebetor*. The moth is considered a model organism in many insect related studies due to its dietetic requirements, environmental adjustability, and growth features. It is a preferred host of many hymenopteran species (Smith, 1965; Burges, 1978; Chang and Hsieh, 1992; Haewoon *et al*., 1995; Coskun *et al*., 2006; Dweck *et al*., 2010).

This study was designed to determine the effect of the venom secreted by females of *B. hebetor*, on the parasitic competence of one of their natural hosts*, G. mellonella*. We hypothesized that venom and other secretions associated with the sting apparatus of parasitoids play a crucial role in the survival and development of parasitoid immatures. Crude venom and different concentrations of diluted venom and Dufour's gland secretions were injected into host larvae to check their mortality or developmental arrest. We transferred eggs from a parasitized host onto an unparasitized host to test the survival and development of non-stung hosts as compared to stung hosts. It provided us basic information related to the activity of host-killing factors present in *B. hebetor,* which will further help us to isolate and characterize the venomous proteins present in female wasp secretions, which may lead towards the development of sustainable biological control programs.

MATERIALS AND METHODS

Experimental conditions

Different life stages of *G. mellonella,* were collected from infested bee hives in different fields present on the campus of the University of Agriculture, Faisalabad (Punjab, Pakistan). The parasitoid and its host cultures were maintained in two separate glass jars placed at 27 ± 1 ˚C and 65 ± 5% RH. The *B. hebetor* culture was maintained at 16/8 h dark-light photoperiod, while *G. mellonella* was reared under complete darkness. The ectophagous parasitic *B. hebetor* was reared on the late larval stage (two day old 5th instar) of *G. mellonella* using a slightly modified approach described by Manzoor *et al*. (2011). Briefly, one to two-day old 5th instar larvae of *G. mellonella* and a single, one-day old adult *B. hebetor* female were placed together in a glass vial (2 x 10 cm) stuffed with cotton pads that were saturated with 50% honey solution as adult food. Prior to oviposition, female wasps started to parasitize the host larva by inserting a minute quantity of venom, which induced partial or complete paralysis of the host larva. When the host larva became sluggish, wasps began egg deposition, which ranged from 3-20 eggs being placed on the outside of the host body. After 24 h, wasps were shifted to new glass vials having two-day old host larvae (5th instar) for parasitization. Hatching of parasitoid eggs took 2–3 days, parasitic larvae fed directly on the exoskeleton of the larval host. Pupation started following completion of the parasitoid feeding period. The parasitoid took nearly 12 days to complete its life cycle (from egg to adult).

Extraction of venom and Dufour's gland secretions

The venom apparatus of *B. hebetor* consists of two glands, the poison (venom) gland (Fig. 1A) and the Dufour's gland (Fig. 1B). The venom gland is linked to a venom reservoir, which is located near to the base of the ovipositor. In order to collect both secretions, *B. hebetor* females, of different ages, were collected into glass vials and refrigerated at -20 °C for 5 min to anaesthetize them prior to the extraction of the venom glands with venom reservoir and Dufour's gland. The reproductive tracts of

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female wasps were removed and placed into a drop of Pringle's saline solution by gripping the tip of the ovipositor with fine forceps while holding the abdomen with another forceps. Venom glands and reservoirs and Dufour's glands were detached from the ovaries and other unwanted tissues with the help of micro dissecting needles. Subsequently, the venom glands and reservoirs were separated from the Dufour's glands and each subset was shifted to a 20 µl drop of chilled Pringle's saline solution.

The venom reservoirs/sacs and the Dufour's glands were gently opened using fine needles to start the removal of their secretions into a drop of saline solution. Almost 20 venom reservoirs or Dufour's gland secretions were added per each 20 µl drop of saline solution. Ultimately, the Pringle's solution drops containing venom or Dufour's glands secretions were transferred to 1.5 ml sterile eppendorf tubes. The resulting crude extracts were centrifuged for 5 min at 5000 g to remove any tissue fragments and the supernatants were shifted to a sterile eppendorf tube and diluted with Pringle's solution to obtain the required concentration of female equivalents per µl of the secretions acquired. Extracted venom or Dufour's gland secretions were either used immediately or stored at -80 °C until use.

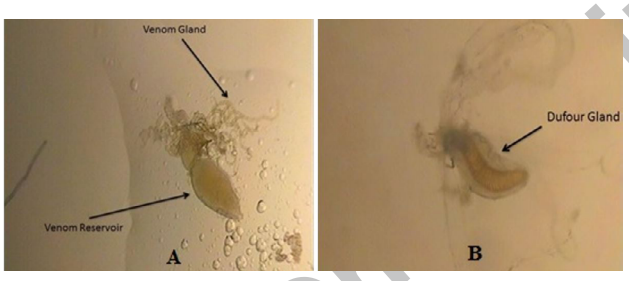

Fig. 1. *Bracon hebetor* sting apparatus showing venom glands with venom reservoir (A) and Dufour's gland (B). Images were taken using digital microscope (40X and 100X Lenses).

Microinjections of B. hebetor *venom and Dufour's gland secretions into host larvae*

We performed two different experiments. First, we evaluated the effect of 1 wasp equivalent mixed secretions (both from venom and Dufour's glands) on the survival and developmental arrest of the host larva, and then we evaluated the effect of different dilutions of both secretions separately on the development of the larval host. Regarding the first set of experiments, two-day old 5th instar *G. mellonella* larvae were divided into four groups on day one of the experiment. Group A (n= 20) was comprised of control larvae of *G. mellonella,* which were separated from the wax and transferred into a petri dish to mature to the adult stage. Group B (n= 20) was comprised of host larvae injected with 1 µl of the Pringle's saline solution to observe the effect of the venom diluent. Group C (n= 20) contained larvae injected using an empty glass micro-capillary tube to observe its purely mechanical effect. Group D (n= 40) larvae were injected with 1 µl venom (1 wasp equivalent/µl). In all cases, host larvae were disinfected by dipping them in a 1.0% ethyl alcohol buffer solution before injection. Larvae were injected using a sterile glass micro-capillary tube and data was recorded at 24 h intervals.

To check the effect of venom and Dufour's gland secretions separately, five dilutions of venom gland secretions were made (*i.e.* 1, 0.5, 0.2, 0.1 and 0.05 wasp equivalent per µl of Pringle's saline solution). Likewise, five dilutions of Dufour's gland secretions were also prepared (*i.e.* 1, 0.5, 0.2, 0.1 and 0.05 wasp equivalent per µl Pringle's saline solution) following the protocol of Rivers *et al*. (1993). Here, one wasp equivalent (1 wasp venom per 1µl Pringle's saline solution) was taken as stock solution and used to make all other required concentrations using Pringle's saline solution as a dilution solvent. Each of the host larvae was injected with 1µl venom solution, which is why we use the term 1 wasp equivalent/µl or 1wasp equivalent/host. For each gland secretion, activity was assayed in two-day old 5th instar *G. mellonella* larvae (n= 15 for each dilution). Experimental insects were anaesthetized on ice for a few minutes and injections containing 1µl of venom or Dufour's gland solution were placed into the pronotum of the host larvae as in Quistad *et al*. (1996) with slightly modifications (Digilio *et al*., 1998, 2000) by using a fine-tipped glass micro-capillary tube. Host larvae injected with 1µl Pringle's saline solution were considered a control treatment. After injection, larvae were kept at 28±1°C and 65±5% relative humidity in darkness and data regarding mortality was recorded at 24 h intervals. The development and paralysis of injected hosts were observed and recorded daily.

Transfer of parasitic wasp eggs onto stung and non-stung hosts

The suitability of stung hosts for parasitic wasp development was tested according to the methodology devised by Cortesero and Monge (1994). Developed male and female parasitoid pairs were confined in transparent glass vials (2 x 10 cm) to obtain a successful mating. On day one, 20 host larvae were stung and paralysed by mated females of different ages, separately in petri-dishes. In a similar way, 20 non-stung hosts were also isolated and kept individually. Parasitoid eggs were removed from stung hosts to subject them to similar accidental mortality factors through handling with forceps. These eggs were divided into two groups: (1) 40 eggs were placed back onto the 20 stung hosts using forceps, with two eggs per host and (2)

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40 eggs were placed onto the 20 non-stung hosts with two eggs per host. All of the eggs were permitted to develop to the adult stage. The survival and development of different life stages of the parasitoid were recorded daily.

Data analysis

Data was statistically analyzed using Statistix 8.1 (Analytical software, 2003) software. Apart from using the basic methods *i.e.* percent distributions, data was analysed using ANOVA and treatment means were separated by a Tukey HSD test at 0.05 level of significance.

RESULTS

The effect of wasp venom injections on host larvae

Most of the un-injected larvae reached the adult stage and there were 80 and 75% larval development in the case of Pringle's saline solution and capillary action, respectively. In the case of venom injection, 32.5% of larvae were killed within one or two days and the remaining 67.5 % did not pass the larval stage and were developmentally arrested (Table I).

Table I. The effect of experimentally injected *Bracon hebetor* venom (1 wasp equivalent/ host) on the development of host (*Galleria mellonella*) larvae.

Treatments	Number of dead host larvae	Number of adult hosts	Successful develop-ment (%)	Develop-mental arrest (%)
Batch A (20)	2	18	90	0
Batch B (20)	4	16	80	0
Batch C (20)	5	15	75	0
Batch D (40)	13	0	0	67.50 (27)

Sample sizes are given in parentheses. Batch A consisted of un-injected control host larvae allowed to develop up to the adult stage. Host larvae from batch B were injected with Pringle's saline solution; whereas larvae from batch C were stung with a glass micro-capillary tube. All of the host larvae from batch D were injected with *B. hebetor* mixed venom diluted with 0.01% Pringle saline solution.

Dose-dependent effect of venom and Dufour's gland secretion on development of host larvae

The results showed that at concentrations of 1.0, 0.5 and 0.2 wasp equivalent/host of the venom gland, 100% mortality occurred, while in the case of 0.1 wasp equivalent, only one larvae out of 15 (6.67%) survived and two larvae (13.33%) receiving 0.05 wasp equivalent successfully developed into adults (Table II). On the other hand, in the case of Dufour's gland activity, mortality of 26.67% (4 out of 15), 20% (3 out of 15), 13.4% (2 out of 15), 6.67% (1 out of 15) and 6.67% (1 out of 15) larvae were recorded with concentrations of 1.0, 0.5, 0.2, 0.1 and 0.05 wasp equivalent/host, respectively (Table II). According to these results, a non-significant mortality was observed in the case of Dufour's gland activity as compared to that of the venom gland, which showed maximum mortality as given in Table II.

Table II. The dose-dependent effect of experimentally injected venom and Dufour's gland secretions of female *Bracon hebetor* on the development of host (*Galleria mellonella*) larvae.

Dose injected (wasp equiva-lent/ host)	Hosts dead at larval stage (%)	Hosts emerged (%)	Successful development (%)
Venom gland			
1(15)	100 ± 0.00 A	0 ± 0.00 B	0 ± 0.00 B
0.5(15)	100 ± 0.00 A	0 ± 0.00 B	0 ± 0.00 B
0.2(15)	100 ± 0.00 A	0 ± 0.00 B	0 ± 0.00 B
0.1(15)	93.33±3.85AB	6.67 ± 3.85 B	6.67 ± 3.85 AB
0.05(15)	86.67 ±3.85 B	13.33±3.85AB	13.33 ± 3.85 A
Control (15)	6.67± 0.00 C	93.33 ± 3.85C	93.33 ± 3.85C
Dufour's gland			
1(15)	26.67±3.85 A	60.00 ±3.85C	60.00 ± C
0.5(15)	20± 3.85 AB	66.67 ±3.85C	66.67 ± C
0.2(15)	13.33±3.85AB	73.33±3.85BC	73.33 ± BC
0.1(15)	6.67±0.00 B	86.67±3.85AB	86.67 ± AB
0.05(15)	6.67± 0.00 B	93.33± 0.00 A	93.33 ± 0.00 A
Control (15)	6.67± 0.00B	93.33±3.85A	93.33 ± 3.85 A

Means followed by different letter(s) within each column (denoted by upper-case letters) are significantly different by Tukey HSD test at P <0.05. Sample sizes are given in parenthesis. Control consisted of host larvae injected with 1µL of Pringle's saline solution.

Developmental characteristics of B. hebetor *eggs transferred onto stung or non-stung hosts*

Development of *B. hebetor* eggs was observed on previously stung (by progenitor female) and non-stung host larvae of *G. mellonella*. Data concerning egg hatching, larval mortality, adult emergence, and mean developmental time of both male and female parasitoids were collected on a daily basis. On stung hosts, 10% of the eggs failed to hatch and 10% were dead at the larval stage, while the remaining 80% developed successfully to the adult stage. The mean developmental time of male and female parasitoids was 9.63 ± 0.38 and 11.13 ± 0.40 days, respectively. In non-stung host larvae, it was observed that 25% of eggs failed to hatch, while the remaining 75% developed successfully up to the adult stage. The mean developmental time of male and female parasitoids was 11.22 ± 0.36 and 12.71 ± 0.31 days, respectively. Male and female development times were significantly different among both treatments (stung and non-stung host larvae) (F=9.30, 9.96; Table III).

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Table III. Developmental characteristics of *Bracon hebetor* eggs transferred onto the host (*Galleria mellonella*) larvae previously stung by a progenitor female or onto the non-stung host larvae.

Treatments	Failure to hatch (%)	Larval mortality (%)	Emerging adults (%)	Mean developmental time of females (days)	Mean developmental time of males (days)
Eggs on stung hosts (40)	10	10	80	11.13 ± 0.40 B	9.63 ± 0.38 B
Eggs on non- stung hosts (40)	25	0	75	12.71 ± 0.31 A	11.22 ± 0.36 A

Sample sizes are given in parenthesis. Means followed by different upper case letters within each column are significantly different (Tukey HSD test; $P < 0.05$).

DISCUSSION

Following micro-injections of venom mixtures (both from venom and Dufour's glands), there was a considerable effect on *G. mellonella* larvae. Mixed secretions caused immediate paralysis as 32.5% larvae died within 5 min of injection. The remaining 67.5% of larvae were developmentally arrested and became flaccid as their feeding on host larvae ceased and death occurred within 24 h. Our findings are in line with the studies of Beard (1978), Piek and Spanjer (1986) and more recently by Moretti and Calvitti (2014) that stated that venoms from almost all of the ectoparasitoid species studied are paralytic, killing their hosts immediately or completely suppressing host development. The small rate of mortality observed in other treatments in our study might be due to the punctures caused by micro-injection and other biotic factors such as post-injection fungal infection.

The effects of different doses of secretions from venom and Dufour's glands showed that the secretions from Dufour's glands alone did not have any significant mortality compared to the venom glands, even at its higher concentrations of 1 wasp equivalent and 0.5 wasp equivalent/host. It shows that these glandular secretions may only have a role in communication as reported by Pickett *et al*. (1982) who postulate that it is a low volatile alarm pheromone and lubricant and not involved in paralysis and death of the host (Gunnison and Morse, 1968; Pickett *et al*., 1982). Gunnison and Morse (1968) stated that since Dufour's gland exits near to the stinger and oviduct, its role could be the secretion of a sting lubricant. Moreover, other functions for this gland are reported in egg coating (Snodgrass, 1925; Trojan, 1930; Dufour, 1835; Mitra, 2013) or as an egg/host marking pheromone (Ratnieks, 1995; Katzav-Gozansky *et al*., 1997b; Martin *et al*., 2002; Mitra, 2013). Similarly, Katzav-Gozansky *et al*. (2003) showed that the Dufour's gland in *Apis mellifera* secretes a series of esters, which function as part of the multi-sourced queen's signal, while in worker bees, the Dufour's gland secretions are eicosenol as reported by Martin *et al*. (2004). This may explain how virgin queens attract workers and workers repel each other by using Dufour's gland secretions as demonstrated by Abdalla and Landim (2001).

In contrast to the Dufour's gland activity, none of the hosts injected with any dilutions of venom gland's secretions completed the larval stage, except for the less concentrated/highest dilutions greater than 0.2 wasp equivalent/host. Highest concentrations (1.0, 0.5 and 0.2 wasp equivalent /µl Pringle's saline solution per host) caused permanent paralysis within 5 min after injection in all hosts causing 100% mortality. This experiment revealed that host death and arrested development observed in the case of mixed secretions was caused by the venom alone, which is secreted by the venom gland, the main source of venom material in *B. hebetor* females. Our results corroborate the findings of Doury *et al*. (1995). The results of our last experiment showed that venom has significant effects on egg hatching and on progeny development of parasitoids, while the larval mortality could be due to rapid manipulation of the host having a weak immune system as different studies show that many parasitoids inject virulence factors into the host during oviposition (Beard, 1978; Stoltz, 1986; Coudron, 1991). Rivers and Denlinger (1994) stated that injected virulence factors subsequently prevent parasitic wasp progeny from being misplaced during development. In the case of un-injected host larvae, once the eggs hatched, no larval mortality was observed. The higher mortality percentages detected for eggs on non-stung hosts may not only be due to the handling of eggs, but also to some response by the immune system of *G. mellonella* larvae (Vinson, 1990).

Both of the parasitoid groups on stung and non-stung hosts exhibited different developmental times. This could be due to the resistance shown by un-injected host larvae to the parasitoid progeny. Although it has been noted that newly emerged *B. hebetor* larvae have the ability to paralyze their larval host not previously stung by a female and showed a successful development on such hosts, either for males or females (Doury *et al*., 1995). Still there could be a difference in development time because in injected hosts, the parasitoid progeny would not face any resistance from the host immune system.

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Our results are supported by the work of Stoltz (1986) who stated that subsequent to hatching of the parasitoid, the next function of the venom may be to help in larval development, either through increasing the nourishing potential of the haemolymph by histolysis or through maintaining the host sanitary state during the stage of host feeding by the parasitic wasp, i.e., through inducing an antifungal or antibacterial action on host haemolymph as manifested by Avolio (2015).

CONCLUSIONS

It could be concluded that *B. hebetor* venom, even in its diluted concentration can result in significant mortality of the host. This could mean that wasps can be manipulated as successful biocontrol agents for lepidopterous insect pests of different crops leading towards the development of sustainable biological control tactics.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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