



## Infection with the insect virus Hz-2v alters mating behavior and pheromone production in female *Helicoverpa zea* moths

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### Abstract

The effect of Hz-2V virus infection on the reproductive physiology and behavior of infected *Helicoverpa zea* female moths was examined. In the absence of males, infected females exhibited calling behavior and called as often but for shorter periods on average than control females. As expected, control females mated with males for extend periods when they were present and did not call after mating, while virus-infected females made many frequent contacts with males and continued to call even after these contacts. Virus-infected females were found to produce five to seven times more pheromone than control females and attracted twice as many males as did control females in flight tunnel experiments. The ability of Hz-2V to alter the physiology and behavior of infected females observed here may serve to facilitate the transmission of virus in insect populations.

**Keywords:** gonad specific virus, *Helicoverpa zea*, Hz-2V, insect virus, mating behavior, pheromone

### Abbreviation:

GSV gonad specific virus

PSP pheromonostatic peptide

### Introduction

A colony of corn earworm moths, *Helicoverpa zea*, from Stoneville, Mississippi was found to be persistently infected with a virus for over five generations (Herzog and Philips 1982; Hamm et al. 1996). This virus was found to replicate exclusively in the reproductive tissues of infected moths and was initially named gonad-specific virus (GSV) (Raina and Adams 1995). Additional work by Hamm et al. (1996) as well as by Rallis and Burand (2002a, 2002b) demonstrated that GSV replicates in a variety of male and female reproductive tissues, including common and lateral oviducts. Hence, the tropism and replication of the virus is not specific for gonadal tissues. This virus is a rod shaped, enveloped virus that resembles Hz-1V in size, pathology *in vitro*, and in genome structure and size (Burand 1998) and, in addition, a comparison of the genome sequence of this virus (Burand unpublished) with the sequence of the genome of the Hz-1V (Cheng et al. 2002) indicates that these two viruses are very closely related. Therefore, in keeping with the present system used to name these types of viruses we propose to name this new virus more appropriately Hz-2V.

Hamm et al. (1996) showed that persistence of Hz-2V in the Stoneville colony was related to the fact that some female moths

in the colony were fertile, asymptomatic carriers of the virus and that these females could vertically transmit the virus to their progeny inside eggs. They also demonstrated that Hz-2V could be horizontally transmitted during mating and suggested that the induction of virus replication and vertical transmission by asymptomatic females was linked to mating.

Hz-2V replication in host reproductive tissues results in the malformation of these tissues and sterility of infected moths, a condition that has been termed agonadal (Raina and Adams 1995). In agonadal females, virus replication occurs primarily in the oviducts leading to the proliferation of cells in these tissues and production of a large number of virus particles that accumulate in association with a matrix of darkly staining material in the cervix bursae. Upon the emergence of these infected female moths, the associated material appears to coalesce around the virus particles and form virus-filled vesicles that make up a clearly visible “virus” plug that can be found covering the tip of the vulva of agonadal females (Rallis and Burand 2002b) and used to easily identify virus infected females. We have recently demonstrated that the “virus” plug can serve as a source of contaminating virus, which healthy males can acquire upon contacting agonadal females and then transmit to other females during subsequent matings (Burand et al. 2004).

The process of finding and attracting suitable mates is the focal point of insect reproductive behavior. As with most insects, the mating pattern of *H. zea* is thought to be regulated by the level of sex pheromone produced by the female. The production of pheromone begins during the first scotophase following adult emergence and peaks during the second and third nights. This peak in pheromone titer triggers female mating and “calling” behavior, which includes rapid wing vibrating, the extruding of the ovipositor and release of sex pheromones via the pheromone gland (Raina et al. 1989; Kingan et al. 1993; Kingan et al. 1995; Raina 1998). This behavior results in the attraction of mates and receptivity to males that attempt to mate by clasping and holding the female’s genitalia with their own (Callahan 1958). After mating, pheromone titers decline and the female loses sexual receptivity due to the transfer of male-derived anti-calling factors, including a pheromonostatic peptide (PSP) (Kingan et al. 1995). This loss of receptivity is only temporary and pheromone production may resume on nights subsequent to the night of mating (Callahan 1958; Kingan et al. 1995).

Interestingly, Raina et al. (2000a) reported that agonadal females vigorously rejected males that attempted to mate. They also state that infected females produced twice the amount of pheromone as control females but failed to substantiate this claim by providing information as to how the pheromone was detected or the amounts of pheromone or pheromone components produced by infected females. It seems somewhat of a paradox that agonadal females would produce a “virus” plug that is able to serve as a source of virus to be transmitted to males (Burand et al. 2004), as well as additional amounts of pheromone to attract males, only to resist mating with them. In order to examine this paradox more closely and to determine if the malformation of reproductive tissues and the presence of this “virus” plug alter the mating behavior of virus-infected female moths, we conducted mating experiments using control and infected females and healthy male moths. The results presented here demonstrate that agonadal females exhibited calling behavior and attracted more male moths in the flight tunnel experiments than do control females. In addition virus infected females were found to produce 5 to 7 times more pheromone than control moths.

## Materials and Methods

### *Insects and virus*

*H. zea* larvae were hatched from eggs obtained from our laboratory colony. Larvae were reared on artificial diet (multi-species diet, Southland Products, Lake Village, Arkansas, USA) in an environmental chamber at 16:8h (L:D) at  $26 \pm 2$  °C and  $50 \pm 5$  % RH. Males and females were separated after pupation and after emergence; adults were provided 10% sucrose solution for feeding.

Hz-2V was purified by sucrose gradient ultracentrifugation as outlined by Burand and Lu (1997). The virus was produced by injecting newly emerged adult female moths with Hz-2V using a micro-applicator (KD Scientific, [www.kdscientific.com](http://www.kdscientific.com)) equipped with a 1 cc syringe and a 26 gage needle. Infected female moths were then placed into individual mating chambers with healthy male moths and allowed to mate. Eggs were collected and larvae reared as described above. Adults from these insects were used as a source

of virus and for use in the experiments outlined below.

### *Female calling and mating behavior*

The calling and mating behavior of newly emerged, virus-infected, female moths was examined during the first scotophase to determine if virus replication resulting in the malformation of reproductive tissues and the presence of a “virus” plug present over the reproductive opening affected the mating behavior of these insects. For female calling and mating experiments, a digital video camera recorder (DCR-TRV830, Sony, [www.sony.com](http://www.sony.com)) was used to observe calling and mating of control and infected moths during two of the first three scotophases. The experiments were conducted in an environmental chamber with a photoperiod of 16:8 h L:D, 60–70% RH, and  $26 \pm 2$  °C of temperature. Newly emerged control or infected female moths (3 per cage) were marked for identification, and then transferred to a Plexiglass cage (15 × 15 × 17 cm) provided with a 10% sucrose solution during the first 2 hours of the photophase preceding the scotophase of observation. The digital video camera recorder was placed approximately 20 cm from the cage and used to record the insects’ calling and mating behaviors. The calling behavior of these females was then recorded during the first and second scotophase in the absence of males. For mating behavior studies 3 males were added to each of the cages and the females’ behavior was observed for the second and third scotophase. Each of these experiments was repeated three times. During both the second and third scotophase after their emergence, the number of times each female called in the presence of males and the duration of each calling period were determined. The number of mating attempts for each female with males, and the length of time each pair was in contact, was also recorded. All variance of calling, mating and mate preference between control and infected moths was analyzed by PROC ANOVA and pairwise mean comparisons made by LSD (SAS Institute 1985).

### *Male preference behavior*

Mate-preference experiments were conducted in a flight tunnel (200 × 75 × 75 cm) made of Plexiglass with window screens at each end to allow for airflow in the chamber (Cardé and Hagaman 1979). For each test, a total of 2 to 4 control or infected female moths at 8–12 h after emergence were held in a small screen chamber, and then placed in the flight tunnel at the upwind end of the flight tunnel in a Plexiglass trap with a funneled opening in one side. The environmental conditions were  $26 \pm 2$  °C,  $50 \pm 5$  % RH, 2.5 lux red light, and 50 cm/s wind velocity. Males were released at the downwind end of the flight tunnel. The total number of males released and the number of replicates for each test are indicated in Table 3. Males collected in the Plexiglass traps were counted after the first or second scotophase and the number of males found in each trap was recorded.

### *Pheromone extraction*

Control adult females and infected adult female *H. zea*, identified by the presence of the “virus” plug were dissected for pheromone extraction at 53–54 hrs post emergence, during the 5<sup>th</sup> hr in scotophase. The moths were taken from environmental chambers and kept in a dark bag until dissection to maintain the dark condition. The moths were removed from the bags, placed on

a waxed dissection dish, and the ovipositors were pushed out from the abdomen using a fine forceps. Since pheromone-gland tissues are located mainly between abdominal segment 8 and 9 (Raina et al. 2000b), insects were cut in the middle of the 8<sup>th</sup> segment with a razor blade and the terminal portion of the abdomen including the ovipositor was used for pheromone extraction. The excised tissues were transferred into gas chromatography sample vials, and soaked for 15 min in 10 µl of ice-cold hexane containing 10 ng/µl of (Z)-7-tridecenyl acetate as an internal standard.

#### Chemical analysis of pheromone extracts

The amount of pheromone produced by tissues samples containing the pheromone glands from control and virus-infected females was examined in an attempt to explain differences observed in the attractiveness of infected females. Each tissue extract (1–3 µl) was analyzed with a Shimadzu ([www.shimadzu.com](http://www.shimadzu.com)) 17A gas chromatograph equipped with a flame ionization detector and an Equity-1 capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; Supelco Inc. [www.sigmaaldrich.com/Brands/Supelco\\_Home.html](http://www.sigmaaldrich.com/Brands/Supelco_Home.html)). Nitrogen was used as the carrier gas. The time for splitless injection was 1.0 min. The oven temperature was set initially at 60 °C for 2 min, increased at 15 °C/min to 250 °C, then 30 °C/min to 300 °C and held for 30 min. The injector and detector temperatures were set at 260 °C and 310 °C, respectively. Septum purge flow rate was set at 3 ml/min and total flow rate at 54 ml/min. The pheromone components were quantified by comparing their peak areas with that of the internal standard, although Z7- and Z9-16:Ald were quantified together since these two isomers could not be resolved using these GC conditions.

## Results

### Calling and mating behaviors

The average number and duration of calls made by control and virus-infected females (which had a “virus” plug) during the first and second scotophase after emergence in the absence of males, and during the second and third scotophases in presence of males is shown in Table 1. The calling behavior of each female was observed during the first and second scotophase in the absence of males to determine if virus infection modified the behavior of these females in any way. As with control females, wing fanning was observed for virus-infected females and appeared normal, however, the presence of the “virus” plug seemed to inhibit the full extrusion and retraction of the ovipositor by infected females. The calling behavior of infected females was most frequent during the period between the 3<sup>rd</sup> and 6<sup>th</sup> hours after the onset of each scotophase, while control insects called at approximately the same frequency throughout the entire scotophase. During the first and second scotophase, in the absence of males, virus-infected females called, on average about as often as controls ( $F = 1.29$   $df = 1$ ,  $p = 0.26$ ). However, the average length of each call by infected females was significantly shorter than those observed for controls with infected females calling an average of 25.01 min compared to an average of 74.36 min for control females during this time period in the absence of males ( $F = 29.89$ ,  $df = 1$ ,  $p = 0.0001$ ).

During the first night in the presence of males, control females made few calls as males often quickly mated with them soon after the scotophase began. No calls by these females were detected on the second night with males present. In these

**Table 1.** Period and number of female calls (mean ± SD) in the absence or presence of males during three continuing scotophases.

Insects	N	1 <sup>st</sup> and 2 <sup>nd</sup> scotophase without males		2 <sup>nd</sup> scotophase with males			3 <sup>rd</sup> scotophase with males		
		Calls	Period (min)	N	Calls	Period (min)	N	Calls	Period (min)
Control	15	26.27 ± 12.92 a*	74.36 ± 31.29 a	9	0.89 ± 1.53	2.24 ± 3.93	9	ND**	ND**
Infected	15	21.06 ± 12.49 a	25.01 ± 16.14 b	9	7.11 ± 3.14	8.90 ± 4.06	9	2.33 ± 1.73	1.79 ± 1.45

\* The same letters in a column are not significantly different ( $\alpha = 0.05$ ), according to least significant difference method (ANOVA, LSD).

\*\* ND indicates that no calling was detected.

**Table 2.** Period and number of female matings (mean ± SD) with males at 2nd and 3rd scotophase.

Insects	N	at 2nd scotophase		at 3rd scotophase	
		Mating #	Period (min)	Mating #	Period (min)
Control	9	1.11 ± 0.33	42.82 ± 39.43	1.11 ± 0.60	103.45 ± 121.3
Infected*	9	9.89 ± 5.71	0.15 ± 0.09	24.02 ± 24.6	0.20 ± 0.10

\* Indicates the number of contacts between infected females and healthy males.

**Table 3.** Total and mean number of males attracted to control and virus-infected females in flight tunnel experiments.

Insects in cages	Number of tests	Total # of males released	# of males respond	Mean % of males in the trap*
Control Females	3	67	50	100
Without Females				0
Control Females	4	154	95	51.49 a
Control Females				48.51 a
Infected Females	3	67	41	50.29 a
Infected Females				49.71 a
Infected Females	10	266	130	64.63 a
Control Females				35.37 b

\* Means within a column followed by the same letter are not significantly different ( $\alpha = 0.05$ ), according to least significant different method (ANOVA, LSD).

**Table 4.** Mean amounts (ng  $\pm$  SD) and ratios of pheromone components in tissue extracts of *H. zea* virus-infected and control virgin females.

Insects	N	Compounds		Ratios of
		Z7 and Z9-16:Ald	Z11-16:Ald	Z11-16:Ald/Z7 and Z9-16:Ald
Control females	8	0.9 $\pm$ 0.24 a*	26.3 $\pm$ 11.3 a	28.8 $\pm$ 7.1 a
Infected females	8	5.3 $\pm$ 2.80 b	185 $\pm$ 91.8 a	35.3 $\pm$ 7.0 a

\* Means within a column followed by different letters are significantly different ( $p < 0.05$ , Kruskal-Wallis tests).

experiments, control females mated only once with the average mating period being between about 1 to 1.5 hours (Table 2), and none of the control females called after mating. In contrast, virus-infected females were observed calling numerous times even with males present (Table 1). These females also called less frequently when males were present since during these experiments it was observed that males made many, brief contacts with virus-infected females as if they were attempting to mate. On average males attempted to mate with virus-infected females about 9 times during the second scotophase, and about 24 times during third scotophase with the average time of these contacts lasting only between 10 to 25 seconds (Table 2).

#### Mate preference behavior

Once it was determined that infected females did exhibit calling behavior, flight-tunnel experiments were conducted to determine if these females were able to attract male moths and if males had a preference for control or virus-infected females (Table 3). In control experiments where females were placed in only one cage it was clear that they were attractive to males since 50 of the 67 insects released were collected in traps with females and none

of the males flew to traps without females. There did not appear to be any bias for one cage or side of the wind tunnel, since when presented with a choice between control females in both cages, or infected females in both, males responded to the two traps with equal frequency. When given a choice between control and infected females, males flew to infected females almost twice as frequently as they did to control females (64.63% compared to 35.37%). This difference is significant ( $p = 0.019$ ) and indicates that virus-infected females are more attractive to males than control females.

#### Pheromone titer

The mean amount of pheromone components found in the tissue extracts of control females were 0.90  $\pm$  0.24 ng/female for Z7/Z9-16:Ald and 26.3  $\pm$  11.3 ng for Z11-16:Ald, whereas those of virus-infected females were 5.3  $\pm$  2.8 ng/female for Z7/Z9-16:Ald and 185  $\pm$  91.8 ng for Z11-16:Ald (Table 4). The differences of mean amounts of each pheromone component between control and infected females were significant ( $p < 0.05$ , Kruskal-Wallis tests), with virus-infected females producing six to seven fold more pheromone than control females. However, the ratio of Z7/Z9-16:Ald and Z11-16:Ald between control and virus-infected females was

not significantly different ( $p > 0.05$ , Kruskal-Wallis tests).

## Discussion

Replication of Hz-2V in the reproductive tissues of *H. zea* results in the malformation of these tissues and sterility of infected moths (Rallis and Burand 2002a, 2002b). In infected females, a large amount of virus accumulates in bursa of pupae, and upon emergence congeals into a “virus” plug over the reproductive opening at the tip of the abdomen. Since this plug has been shown to be a source of contaminating virus for male moths that attempt to mate with infected females, we examined the behavior of female moths to determine if virus infection and the presence of this plug affected mating and calling of infected insects.

In studies where female behavior was observed in the absence of males, infected females called as often as controls but for significantly shorter periods than control females. This suggests that the shorter length of calls may be a result of a decrease in the fitness of virus-infected females compared to controls. In the presence of males control females displayed the expected very short (sometime undetected) bouts of calling and mated only once a night, while the calling bouts by infected females were significantly more frequent and for longer periods.

During these experiments actual contacts between males and virus-infected females did take place and although brief (only 10 to 20 sec each) these contacts between pairs were quite common. Actual clasping of agonadal females by males was observed but was very infrequent. While control females were never observed to call after mating most of the virus-infected females resumed calling after being in contact with the males. This was expected since the “virus” plug found at the tip of the abdomen of agonadal females acts like a mating plug (Orr 1995), preventing the transfer of PSP and other inhibitors of calling to virus infected females.

As observed by Raina et al. (2000a), agonadal females appear to reject males that make an unsuccessful attempt to grab and clasp them. The short contacts between males and virus-infected females observed in this study may be the result of the “virus” plug interfering with the male’s ability to clasp and hold the genitalia of agonadal females. These contacts, although brief, could serve to contaminate males, which have been shown to be able to transmit virus to females during subsequent matings (Burand et al. 2004).

In flight tunnel experiments, agonadal females attracted twice as many males on average as did control females. This increase in the attractiveness of infected females appears to be the result of an increase in the level of pheromone produced by agonadal females as suggested by Raina et al. (2000a). Our analysis of the pheromone produced by females revealed that infected females produced as much as 5 to 7 times more Z7/Z9-16 Ald and Z11-16 Ald than did control females. It appears that this increase in pheromone makes virus-infected females more attractive to potential mates and possibly contributes to the spread of virus among insects through the contamination of male moths that attempt to mate with these females.

It is interesting to find that virus replication can alter the reproductive physiology and behavior of infected female moths. However, this is not too surprising considering that the primary site of replication of Hz-2V is in insect reproductive tissues. The results presented here suggest that the virus has evolved such that its

transmission has become linked with host reproductive physiology and behavior. The behavior of males making frequent short contacts with infected females may also be a consequence of this evolution. The increase in the attractiveness of infected females and the frequent contacts between these insects and control males may actually play an important role in facilitating the transmission of the virus in insect populations.

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