

Mate Preference and Mating Behavior of Male *Helicoverpa zea* (Lepidoptera: Noctuidae) Infected with the Sexually Transmitted Insect Virus Hz-2V

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ABSTRACT The affect of Hz-2V (a.k.a. gonad-specific virus) virus infection on the reproductive physiology and behavior of infected adult *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) males was examined. Virus-infected males responded and flew to calling healthy and infected females at about the same rate. Although infected males were slower in approaching healthy females and attempting to mate compared with healthy males, infected males did make sexual contacts with females that could serve to transmit virus. Unlike matings with healthy males, which result in the cessation of calling, females that had made sexual contact with infected males continued to exhibit calling behavior and were receptive to mates. Although calling behavior of both healthy and infected females was inhibited by injecting extracts of reproductive tissues from healthy males, extracts of reproductive tissues from infected males inhibited female calling to a lesser degree and for an additional night compared with extracts from healthy males. The results presented here further demonstrate the ability of Hz-2V to alter the physiology and behavior of infected insects in ways that could serve to facilitate virus transmission.

KEY WORDS Hz-2V, gonad-specific virus, pheromonostatic peptide, mating behavior, *Helicoverpa zea*

Hz-2V (a.k.a. gonad-specific virus [GSV]) is a sexually transmitted insect virus that replicates in the reproductive tissues of both male and female insects, resulting in the sterility of infected adults (Hamm et al. 1996). This virus was initially found in a colony of corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), originating from the USDA-ARS laboratory in Stoneville, MS (Herzog and Phillips 1982, Raina and Adams 1995, Hamm et al. 1996) and was subsequently reported in four feral populations of this moth (Raina et al. 2000). In females, productive Hz-2V replication occurs mainly in the common and lateral oviducts culminating in the production of copious amounts of virus that is released into the lumen of the oviducts and accumulates in a plug covering the reproductive opening of the adult female (Hamm et al. 1996, Raina et al. 2000, Rallis and Burand 2002b). This "virus" plug at the tip of the female's abdomen then serves as a source of virus for males, which become contaminated while attempting to mate, and these males ultimately transfer the virus to healthy females during subsequent mating attempts (Burand et al. 2004).

Hz-2V infection results in males that are missing or have underdeveloped testes, lack accessory glands, and have grossly malformed seminal vesicles, vasa deferentia, and duplex and simplex, but they retain an aedeagus and endophallus that seems normal and functional (Hamm et al. 1996, Raina et al. 2000, Rallis and Burand 2002a), suggesting that sterile, virus-infected males may transmit virus to healthy females during copulation.

Hz-2V replication in *H. zea* females results in altered mate calling behavior, increased sex pheromone production and the attraction of more males than healthy females (Burand et al. 2005). In the current study, we examined the reproductive behavior of virus-infected males to determine whether these insects show a preference for healthy or infected females and to determine whether actual mating attempts by infected males occur. Because anti-calling factors, some of which are produced by the male accessory glands, which are absent in virus-infected insects, are transferred during mating by healthy adult moths (Kingan et al. 1993), extracts of reproductive tissues from healthy and infected males were examined for their ability to inhibit calling behavior of healthy and infected females. The results presented here indicate that, as with infected females, Hz-2V replication in male moths results in changes in the reproductive physiology and behavior of these insects in ways that

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could serve to facilitate virus transmission between insects during mating attempts.

Materials and Methods

Insects and Virus. The *H. zea* colony was maintained as described previously by Burand et al. (2005), and Hz-2V was purified by sucrose gradient ultracentrifugation as outlined by Burand and Lu (1997). Newly emerged female moths were injected with $>2 \times 10^6$ tissue culture infective dose₅₀ units of Hz-2V. We previously demonstrated that at this virus concentration, 100% of the progeny hatching from eggs laid on the third day after mating show viral pathology (Burand and Rallis 2004). Injections were performed using a microapplicator (KD Scientific, Holliston, MA) equipped with a 1-cc syringe and a 26-gauge needle. Infected female moths were then placed into individual mating chambers with healthy male moths and allowed to mate. Eggs laid on the third day after mating were collected, and larvae were reared on artificial diet (Multi-species diet, Southland Products, Lake Village, AR) held at $26 \pm 2^\circ\text{C}$, 60–70% RH, and a photoperiod of 16:8 (L:D) h. After pupation, males and females were separated, and adults from these insects were used in the experiments outlined below.

Calling and Mating Behavior. The calling and mating behavior of newly emerged, healthy female moths were examined in the presence of virus-infected male moths. Two female moths were marked for identification and transferred to a Plexiglas cage (15 by 15 by 17 cm) with four healthy or virus-infected male moths and a 10% sucrose solution. A digital videocamera (Sony DCR-TRV830) was used to record calling and mating behavior during the first two scotophases. The experiments were conducted in an environmental chamber as described above and repeated four times with the number and period of each female calling and mating determined from recordings made using the videocamera.

Virus-Infected Male Preference Behavior. Virus-infected male preference experiments were done in a flight tunnel (200 by 75 by 75 cm) made of Plexiglas with window screens at each end to allow airflow in the chamber (Cardé and Hagan 1979). Four healthy or virus-infected female moths, 8–12 h after emergence, were held in a small screen chamber, which was placed in a Plexiglas trap with a funnel opening in one side and then located at the upwind end of the flight tunnel. The environmental conditions were $26 \pm 2^\circ\text{C}$, $50 \pm 5\%$ RH, 2.5-lux red light, and 50 cm/s wind velocity. For each experiment, ≈ 20 virus-infected male moths were released at the downwind end of the flight tunnel. The male moths collected in the Plexiglas traps were recorded after the first or second scotophase.

Male Accessory Gland Extracts. Male accessory glands were obtained from both 2-d-old virgin healthy and infected males, and extracts were prepared following the methods of Delisle et al. (2000) and Kingan et al. (1993). A set of 10 accessory sex glands from healthy or infected males were removed under a saline

Table 1. Behavior responses of infected male *H. zea* to healthy female *H. zea*

Cages with insects	No. testes	Total no. of males released	No. males responded	Mean % males in the cage ^a
Healthy females	6	116	66	100
Without females				0
Healthy females	3	48	22	50.73a
Healthy females				49.27a
Infected females	5	95	60	50.62a
Healthy females				49.38a

^a Means followed by the same letter are not significantly different ($\alpha = 0.05$), according to LSD test (ANOVA).

solution and immediately transferred into a 1.5-ml microcentrifuge tube on ice. The glands were homogenized in 50 μl of saline and centrifuged at 4°C for 10 min at $1,000 \times g$ to give a concentration of 1 accessory sex gland per 5.0 μl . Newly emerged healthy or infected female moths were then injected with 5.0 μl of these accessory sex gland homogenates at ≈ 2 h before the scotophase to investigate what effect these male extracts had on female calling behavior. Female moths injected with 5.0 μl of the saline solution or injected with virus as outlined above were used as controls. Observations of the calling behavior of these female moths were conducted as described above.

Data Analyses. All variance of calling, mating, and male preference between healthy and virus-infected moths was analyzed by PROC analysis of variance (ANOVA), and pairwise mean comparisons made by least significant difference (LSD) test (SAS Institute 1998).

Results

Mate Preference Behavior. Flight tunnel experiments were conducted using virus-infected male moths to determine whether Hz-2V infection of these insects resulted in them having a preference for infected or healthy females. When healthy females were placed in a cage in only one side of flight tunnel, 66 of the 116 (57%) males released were collected in traps with the caged female, and no males were collected in traps without any females in the cage (Table 1). When healthy females were placed in cages on both sides of one end of the flight tunnel, 46% of the infected males flew to cages with females, and they flew to cages on both sides of flight tunnel at the same rate (50.73 compared with 49.27%). In experiments where infected males were offered a choice between healthy or infected females, they showed no preference for either category of females and flew to cages with infected females at almost the same rate as they flew to cages with healthy females.

Calling and Mating. When females were placed in the mating chamber with infected males, they called between 2 and 5 times more frequently as when healthy males were present, and each calling bout

Table 2. Period and number of female *H. zea* calls with healthy or infected male *H. zea* during two continuing scotophases

Insects	<i>n</i> ^a	First scotophase ^b		Second scotophase ^b	
		No. calls	Period (min)	No. calls	Period (min)
HF × HM	6	6.83 ± 8.2a	5.36 ± 6.4a	3.50 ± 4.0b	2.74 ± 2.9b
HF × IM	8	16.50 ± 12.0a	28.42 ± 41.1a	15.87 ± 12.0a	26.22 ± 28.5a

Data are mean ± SD. HF, healthy females; HM, healthy males; IM, infected males.

^a Numbers of females used in the experiment.

^b Means followed by the same letter are not significantly different ($\alpha = 0.05$), according to LSD method (ANOVA).

lasted ≈5–10 times as long as did those in the presence of healthy males (Table 2). Although the differences in the number and the duration of bouts of calling by females when in the presence of healthy or infected males were not statistically significant on the first ($F = 2$, $df = 1$, $P = 0.11$; $F = 1.82$, $df = 1$, $P = 0.2$) night, they were significantly different on the second night ($F = 5.77$, $df = 1$, $P = 0.03$; $F = 3.95$, $df = 1$, $P = 0.04$).

In these mating experiments healthy males were observed to quickly approach females, and as they approached the female, they twisted their abdomen at an angle that positioned it closer to the female’s abdomen. They then extended their claspers and locked the female’s genitalia with their own. In cages where healthy males were present, each female mated once a night for periods from 1 to 2 h (Table 3), and no female was observed to exhibit calling behavior in the same scotophase after mating with a healthy male.

Virus-infected males reacted much more slowly to female calls than did their healthy counterparts. The infected males seemed less deliberate when approaching females and were inaccurate in >90% of their attempts to grab and clasp females that had been calling. The females seemed to become mildly agitated by these imprecise advances by infected males and often flew some distance away. During these experiments, only one successful attempt by an infected male to clasp a female was observed, and this mating attempt lasted only ≈1 min, after which the female was observed to call 24 more times. Each of the eight females in these experiments were observed making, on average, 5.0 brief contacts with males during the first night and, on average, 6.3 contacts during the second night.

Response of Females to Male Reproductive Tissue Extracts. Control healthy and infected females injected with saline alone continued to call (9.00 ± 2.2 and 7.87 ± 5.4 calls, respectively), whereas females injected with extracts from the reproductive tissue of

healthy males called very infrequently, with injected healthy females calling less than once (0.44 ± 0.9) and infected females making 1.5 calls (1.50 ± 2.2) after being injected with healthy male extracts (Table 4). Healthy females injected with extracts from infected males called more frequently than females injected with healthy male extracts, averaging 4.91 ± 1.1 times per night. The mean number of calls by both healthy and infected females, on the night after the treatment (6.89 ± 4.3 and 7.82 ± 7.0 , respectively), returned to the same level as that of the healthy (6.38 ± 3.2), saline-treated controls, whereas healthy females treated with extracts from infected males called significantly less, averaging only 2.54 ± 0.7 calls on the night after treatment. Healthy females injected with virus called at the same rate as the saline controls both on the night of treatment (7.75 ± 8.5 and 9.00 ± 2.2 calls, respectively) and on the night after treatment during which time injected females called 11.05 ± 10.2 times compared with 6.38 ± 3.2 calls for the controls.

Discussion

In their initial characterization of Hz-2V, Hamm et al. (1996) reported that although no infected progeny were produced from individual mating pairs of virus-infected males and healthy females, mass matings of infected males and healthy females for only one night did result in infected progeny. Because of the transfer of anti-calling factors from the males during mating, *H. zea* females do not normally call and are not receptive to males on the same night after mating (Callahan 1958, Raina et al. 1994). Therefore, Hamm’s results suggest that virus-infected progeny arise from healthy females that had acquired the virus from sterile, infected males while attempting to mate and that these newly infected females remain receptive to healthy males, which mated and fertilized them.

Table 3. Period and number of female *H. zea* mating with healthy or infected male *H. zea* during two continuing scotophases

Insects	<i>n</i> ^a	First scotophase ^b		Second scotophase ^b	
		No. mating	Mean period (min)	No. mating	Mean period (min)
HF × HM	6	1.83 ± 1.4a	73.36 ± 52.1a	1.50 ± 0.38a	127.57 ± 112.2a
HF × IM	8	0.00 ± 0.0b	0.00 ± 0.0b	0.25 ± 0.46b	0.15 ± 0.36b

Data are mean ± SD. HF, healthy females; HM, healthy males; IM, infected males.

^a Numbers of females used in the experiment.

^b Means followed by the same letter are not significantly different ($\alpha = 0.05$), according to LSD method (ANOVA).

Table 4. Period and number of female *H. zea* calls injected with supernatant solution of male reproductive tissues

Insects ^a	n ^b	First scotophase ^c		Second scotophase ^c	
		No. calls	Mean period (min)	No. calls	Mean period (min)
SaInHf	8	9.00 ± 2.2a	8.98 ± 2.1a	6.38 ± 3.2bc	10.16 ± 2.2a
SaInVf	16	7.87 ± 5.4ab	5.46 ± 2.5ab	15.00 ± 4.9a	8.24 ± 4.6abc
HmInHf	18	0.44 ± 0.9d	0.18 ± 0.2d	6.89 ± 4.3bc	5.13 ± 3.8bcd
HmInVf	28	1.50 ± 2.2cd	1.04 ± 1.2cd	7.82 ± 7.0bc	4.37 ± 2.5cd
VmInHf	11	4.91 ± 1.1bc	4.40 ± 1.5bc	2.54 ± 0.7c	2.36 ± 1.1d
VpInHf	20	7.75 ± 8.5ab	8.08 ± 10.6ab	11.05 ± 10.2ab	9.00 ± 7.3ab

^a SaInHf, healthy female was injected with supernatant solution of saline buffer; SaInVf, virus-infected female was injected with supernatant solution of saline buffer; AmInAf, healthy female was injected with supernatant solution of reproductive tissues from a virus-infected male; HmInAf, virus-infected female was injected with supernatant solution of reproductive tissues from healthy male; HmInNf, healthy female was injected with supernatant solution of reproductive tissues from healthy male; and VpInNf, healthy female was injected with purified Hz-2V.

^b Numbers of females used in the experiment.

^c Means followed by the same letter are not significantly different ($\alpha = 0.05$), according to LSD method (ANOVA).

For healthy *H. zea*, when a male successfully locks with a female the pair begins copulating, and in the process, the male transfers sperm and male reproductive secretions into the bursa copulatrix of the female. One component of the seminal fluid transferred to the female during copulation is the anti-calling factor pheromonostatic peptide (PSP). PSP is produced by the accessory gland of the male and when transferred to the female during mating leads to a drop in pheromone titer and the cessation of calling of mated females (Raina et al. 1989, Kingan et al. 1995, Raina 1998). The ability of Hz-2V infected males to transmit virus to healthy females without altering their receptivity seems possible, because these males lack accessory glands and probably do not produce PSP (Raina et al. 1989, Raina 1998).

To determine whether virus-infected males respond to healthy, calling females and to observe how healthy females responded to infected males, flight tunnel and mating experiments were conducted as described previously for healthy males and virus-infected females (Burand et al. 2005). When placed in the flight tunnel, 57% of the virus-infected males responded to females and flew to cages containing females. This rate was about the same as that reported for healthy males flying to females (Burand et al. 2005). However, unlike healthy males that flew to virus-infected females at about twice the rate as they flew to healthy females, infected males did not demonstrate a preference for infected females and flew to healthy and infected females at about the same rate.

In mating experiments, infected males were observed to frequently approach calling females, twist their abdomens toward the female, and make sexual contact with the female as they attempted to clasp and hold her. These attempts to mate by infected males seemed to agitate the female, which in response flew some distance away from the male. These rejected advances included brief, sexual contacts between mating pairs that lasted only 1–2 s but could be long enough for the virus to be transmitted between the pair. The difference in the number and the length of calling bouts by healthy females observed in these experiments occurred because healthy males re-

sponded more quickly to female calls than did infected males.

Hz-2V-infected males have been shown to lack accessory glands and therefore are thought not to be able to produce and transfer PSP to females (Rallis and Burand 2002a). Because we did observe a short mating attempt by a virus-infected male with a female that continued to call, we began to investigate whether male reproductive fluids from virus-infected males contained any anti-calling factors. We also investigated whether virus-infected females, which had previously been found to continue to call after mating attempts with healthy males (Burand et al. 2005), were able to respond to anti-calling factors from healthy males and stop calling.

The injection of extracts of reproductive tissues from healthy males into virus-infected females reduced the number of times these females called to the same level as healthy females injected with these extracts. Because it has been shown that infected females continue to call after mating attempts by healthy males (Burand et al. 2005), this result demonstrates that infected females are able to respond to anti-calling factors and also illustrates that the "virus" plug covering the reproductive opening of these females acts like a "mating" plug (Orr 1995), serving to block the transfer of anti-calling factors to these females by healthy males that attempt to mate with them. Thus, these females continue to call after having sexual contacts with healthy males, further facilitating virus transmission by attracting and contaminating more potential mates.

Hz-2V-infected males do seem to produce some type of anti-calling factor(s) because extracts of the reproductive tissues from these males reduces the rate of calling of healthy females compared with saline-injected controls but not to the same level as extracts from healthy males. Compared with extracts from healthy males, which reduced female calling only on the night of injection, extracts of from infected male tissues reduced female calling on the night after injections as well. The anti-calling factor(s) from infected males is probably different from those produced by their healthy counterparts because it did not

reduce calling to the same level as did extracts of reproductive tissues of healthy males and because its effects were longer lasting. Because Hz-2V-infected males lack accessory glands, it is unlikely that extracts from these males contain the anti-calling factor PSP, which is produced by these tissues in healthy males. One possibility is that the anti-calling factor from the infected male is a product of virus replication in the infected insect. This is unlikely, however, because females injected with purified virus called at the same rate as the saline controls.

The results presented in this study further demonstrate how the sexually transmitted insect virus, Hz-2V has evolved to alter the physiology and mating behavior of infected insects in ways that facilitate virus transmission between insects. In infected females, which are more attractive to prospective mates than their healthy counterparts), Hz-2V replication culminates in the formation of a plug of virus covering the reproductive opening of these insects, which serves both as a source of virus for males attempting to mate and as a barrier to the transfer of anti-calling factors, including PSP, from these males. Consequently, these females, which are capable of responding to anti-calling factors produced by healthy males, continue to call after sexual contact with males, attracting and contaminating additional prospective mates.

We also report that Hz-2V-infected males do approach and make sexual contact with healthy females that continue to call and attract potential mates. Although imprecise and lasting only a short time compared with those between healthy mating pairs, these contacts are almost certainly long enough for virus transmission to take place and help to explain the results of mass-mating experiments of Hamm et al. (1996). It is interesting that the altered reproductive behavior that we have shown to occur in mating attempts involving moths infected with this sexually transmitted virus results in both the infected moth and its contaminated mate being able to come in contact with more potential mates, with additional mating attempts further increasing the probability and rate of spread of the virus among individuals in the population.

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