Pathology and transmission of Hz-2V infecting the reproductive tissues of the corn earworm, Helicoverpa zea.

PATHOLOGY AND TRANSMISSION OF Hz-2V INFECTING THE REPRODUCTIVE TISSUES OF THE CORN EARWORM, *Helicoverpa zea*.

A Thesis Presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

February 2006

Entomology

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ACKNOWLEDGEMENT

I would like to thank my advisor, John P. Burand, for his many years of patience, guidance, and support. I would also like to thank my committee members, Adam Porter and Joseph Kunkel, for their insight and assistance in making this thesis possible. Many thanks also to Chih-Ming Yin, who advised me and helped me in various aspects of my research, and to the rest of the faculty and office staff of the Department of Entomology for their assistance in making this project possible.

I want to thank those who helped fund this research, provided in part by the Lotta Crabtree Graduate Fellowship in Agriculture, by USDA NRICGP grant #2001-35302-10885, and by Project # MAS00802 of the Massachusetts Agricultural Experiment Station.

I would like to gratefully acknowledge the generous gift of insects and virus from Dr. J. Hamm (USDA), the services of Lucy Yin and the UMass Central Microscopy Facility, which is supported in part by Grant NSF-BBS-87-14235, the assistance of Wendy J. Hughes and Heather Valley in maintaining the insect colonies and performing dissections, and the technical assistance of Woojin Kim, Sarah Carpenter, and Dr. Meng-Ping Tu.

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LIST OF ABBREVIATIONS

ATOB	atypical occlusion bodies
CAV	cell-associated virus
dpp	days post pupation
ECV	extracellular virus
ENC	enveloped nucleocapsid
GSV	gonad-specific virus
H. zea	*Helicoverpa zea*
NC	nucleocapsid
PBS	phosphate-buffered saline
pfu	plaque-forming units
PSP-1	pheromonostatic peptide, PSP-1
$TCID_{50}$	tissue culture infective dose 50
Hz-2V	virus' current name (formerly GSV)

CHAPTER 1

GENERAL INTRODUCTION

Discovery and Pathology of Hz-2V

At the USDA-ARS in Stoneville, MI, it was noticed that a colony of corn earworm moths, *Helicoverpa zea*, was producing fewer viable eggs than normal. Herzog and Philips (1982) examined the reproductive tissues of the affected moths, found that the reproductive tissues of some of the adults were grossly malformed, and attributed these malformations to a genetic condition. However, several years later, Raina and Adams (1995) and Hamm *et al.* (1996) re-examined the abnormal tissues of infected moths and found that the malformed tissues were infected with a rod shaped, enveloped virus.

Interestingly, although the virus was found in the reproductive tissues, the virus could not be detected in fat body, muscle, tracheal matrix, or any other tissue, nor could the virus be found in any other life stage of the corn earworm. Because the virus was found only in the adult reproductive tissues of both male and female moths, causing gross malformation of these tissues and resulting in the insects being sterile, the virus was designated gonad-specific virus, or GSV (Raina and Adams 1995). However, because the virus is not limited to the gonads, the virus was renamed Hz-2V, according to current virus nomenclature (Burand 1998).

Hamm *et al.* (1996) and Raina *et al.* (1995, 2000) reported that the reproductive tissues of the infected female moths were malformed into a Y-shaped structure and a smaller C-shaped structure, and the ovaries, bursa copulatrix, accessory glands, and spermatheca were completely absent. A "waxy plug" was usually observed covering the vulva of infected female moths. In infected male moths, the reproductive tissues were

also grossly malformed, and the seminal vesicles, vasa deferentia, and accessory glands were absent. Because much of the reproductive tissues in these infected, sterile moths were absent, these moths were designated as being agonadal (Raina and Adams 1995) and (Hamm *et al.* 1996).

In ultrastructural examinations of the malformed tissues, extremely hypertrophied epithelial cells were found containing grossly enlarged nuclei filled with virus particles arranged in numerous arrays (Raina *et al.* 1995, 2000) and (Hamm *et al*, 1996). Virus particles were also found in intercellular spaces and in the lumina of the malformed reproductive tissues, and the "waxy plugs" typically found over the vulva of agonadal female moths were filled with large quantities of virus particles contained in vesicles that have been called atypical occlusion bodies (ATOB) (Hamm *et al*, 1996).

Detailed electron microscopic examination of Hz-2V revealed that it is a rod shaped, enveloped virus averaging 415 x 81 nm, with the nucleocapsid averaging 381 x 52 nm. Cross-section of virus particles revealed that the envelope is loosely associated with the nucleocapsid (NC) and folded into several ridges or shallow pleats, with a single small filament associated with each ridge (Hamm *et al.* 1996).

***In vitro* Replication**

Reflective of Hz-2V's affinity for reproductive tissues, the virus has only been successfully maintained *in vitro* in cell lines established from ovarian tissues. To date, only the ovarian cell lines Tn-368 cells established from the cabbage looper, *Trichoplusia ni* (Burand and Lu 1997) and (Lu and Burand 2001), Ld652Y cells from the gypsy moth, *Lymantria dispar* (Lu and Burand 2001), and IPLB-HvT1 cells from the tobacco

budworm, *Heliothis virescens* (Raina *et al.* 2000) have been used successfully to study replication of Hz-2V *in vitro*. Data from studies of Hz-2V replication *in vitro* in Tn-368 cells revealed that Hz-2V is heterogeneous and that the virus isolated from these cells is genetically identical to the virus isolated from reproductive tissues in *H. zea*. In both the Tn-368 and the Ld652Y cell lines, Hz-2V exhibits a rapid increase in cell-associated virus (CAV) followed by a rapid increase in extracellular virus (ECV). However, in Ld652Y cells Hz-2V replication was more productive, formed viral plaques, and assembled virus in arrays inside the nuclei of infected cells as is seen *in vivo* (Lu and Burand 2001). In addition, virus particles in small vesicles were found associated with infected Ld652Y cells and were thought to be budding from the cells, as was reported to occur *in vivo* (Raina and Adams 1995). Yet even Hz-2V replication in these cells did not exactly match virus replication *in vivo* in the corn earworm, suggesting that some signals or cues were absent *in vitro*.

Virus Transmission

In detailed transmission studies, Hamm *et al.* (1996) conducted experimental crosses between moths from the infected Stoneville, MI colony and healthy moths from a USDA-ARS Tifton, GA colony of *H. zea*. The progeny resulting from these crosses were examined for agonadal pathology, and some were also examined ultra-structurally for the presence of virus particles. Stoneville females mated singly to Tifton males, and Stoneville females mated singly to Stoneville males produced agonadal progeny, indicating that these Stoneville females were apparently normal, fertile, asymptomatic carriers of Hz-2V, and were able to transmit the virus to their progeny. The reproductive

tissues of some apparently normal male and female moths were found to contain virus particles, confirming the presence of these carrier moths.

In the only evidence of horizontal transmission of Hz-2V during mating, Tifton females mass-mated for one night with Stoneville males produced agonadal progeny in Hamm's *et al.* transmission experiments (1996), suggesting that during mating Stoneville males are able to transmit Hz-2V to healthy females, which are then able to transmit the virus to their resulting progeny. Although Tifton females mated individually to Stoneville males produced no agonadal progeny, the apparently normal progeny were not tested for the presence of Hz-2V, and it is possible that some may have been asymptomatic carriers of the virus.

In further transmission studies, Hamm *et al.* (1996) inoculated different life stages of the corn earworm with various results. A low level of transmission, 5.3%, was achieved by treating eggs with virus, whereas injecting larvae with Hz-2V resulted in a high incidence of infection, with 100% of all females being agonadal. Adult moths fed a suspension of the virus-filled "waxy plug" produced 26.2% agonadal progeny. More impressive still, adult female moths injected with Hz-2V into the abdomen and then mated to uninfected males produced up to 100% agonadal progeny.

These artificial methods of Hz-2V transmission also proved beneficial for propagating the virus *in vivo*. Although Hz-2V could be propagated *in vitro*, the virus was most productive *in vivo*, harvested from the reproductive tissues and "waxy plugs" of progeny agonadal female moths resulting from eggs laid by females injected with Hz-2V as adult moths.

Dose-Response

In Hamm's transmission experiments, he found that in all cases in which agonadal progeny were produced, the percentage of progeny that were agonadal increased with increasing oviposition day (Hamm *et al.* 1996). Yet, since the moths were only allowed to mate for one scotophase, it would seem that any contamination from the infected adults should be greatest on the earliest oviposition days. This suggests that the virus may have increased its infectious titer through replication in the reproductive tissues of the infected parent female and then been transferred in increasing doses to progeny insects inside eggs. These data from agonadal progeny, in addition to the data confirming the presence of fertile asymptomatic carriers of Hz-2V, led Hamm to suggest that the virus dose transmitted to the eggs from the infected female moth affects the outcome of the infection, resulting in agonadal or asymptomatic carrier moths. He suggested the earliest progeny do not receive an infectious dose of virus, whereas still others receive a dose too small to produce the agonadal condition, yet sufficient virus to become asymptomatic carriers of Hz-2V. He also suggested that the progeny resulting from eggs laid on the latter oviposition days receive the largest doses of infectious virus, resulting in the agonadal condition in their progeny insects.

In addition, the data from a dose-response experiment conducted by Raina *et al.* (2000) indicate the initial infectious dose of Hz-2V received by a female moth affects the outcome of the infection in progeny moths. The data revealed that the percent agonadal of the total progeny resulting from eggs laid by an infected female moth increased with increasing dose received by the infected female. Asymptomatic carriers were not examined in Raina's experiment.

Objective of This Research

These data indicate that Hz-2V has tremendous potential as a biological control agent of *H. zea*. Severe infections of the virus result in the sterilization of both male and female moths, yet the virus is also able to establish persistent infections in other life stages of *H. zea* and in asymptomatic carrier moths. However, we don't know the exact nature or mechanism of the malformations of the reproductive tissues caused by the virus, and we know little about Hz-2V replication *in vivo*.

The pathology and ultrastructure of Hz-2V infecting agonadal male and female corn earworms were examined in order to determine the exact changes wrought by the virus in the development and differentiation of the reproductive tissues of infected moths, and the effect of virus infection on reproductive behavior and virus transmission. In addition, experiments were conducted to examine the ability of agonadal moths to mate with and transmit Hz-2V to uninfected mates, and the ability of these newly infected moths to further transmit the virus to their progeny resulting from subsequent matings with uninfected, fertile moths.

Just as these agonadal moths may play a key role in horizontal transmission of Hz-2V to healthy mates, asymptomatic carrier moths likely play a key role in virus persistence within the population of corn earworm moths. The relationship between the two different outcomes of infection with Hz-2V, resulting in agonadal and asymptomatic carrier moths, was examined in detail in order to better understand why or how this dual infection strategy has evolved in *H. zea* and what affect this strategy has on the virus-host ecology.

CHAPTER 2

HZ-2V INFECTION IN MALE *HELICOVERPA ZEA*

Introduction

The pathology of Hz-2V [a.k.a. gonad-specific virus (GSV)] infecting the reproductive tissues of corn earworm moths, *Helicoverpa zea*, originating from a colony of insects at the USDA-ARS in Stoneville, MS has been described by Hamm *et al.* (1996) and Raina *et al.* (1995, 2000). Replication of this virus appears to be limited to the reproductive tissues of both male and female moths, causing gross malformation of some of these tissues, resulting in infected moths being sterile. Since much of the reproductive tissues in these infected, sterile moths are absent, these insects have been described as being agonadal. The results of experimental matings between healthy moths and moths from the Stoneville colony (Hamm *et al.* 1996) indicate that infected insects can vertically transmit Hz-2V and that some virus infected female moths are fertile, asymptomatic carriers of the virus.

Interestingly, in these experiments (Hamm *et al.* 1996) individual matings of healthy females with Stoneville males resulted in no infected progeny, yet healthy females mass-mated with Stoneville males for the duration of one scotophase produced some agonadal progeny. Since both asymptomatic and agonadal moths can carry the virus, these results suggest that one or both of these two types of virus-infected, male moths are capable of horizontal transmission of Hz-2V. Since the mass matings were allowed to occur for only one scotophase, females that mated with sterile agonadal males and produced viable infected progeny must have also mated with a healthy male during the same scotophase. Normally, female moths mate only once during each scotophase

and lose sexual receptivity due to male-derived anti-calling factors and a pheromonostatic peptide (PSP-1) secreted by the reproductive system of normal male moths and transmitted to females during copulation (Kingan *et al.* 1993, 1995; Raina *et al.* 1994).

In order to determine if agonadal male moths are capable of transmitting Hz-2V to healthy females during mating, we examined the reproductive tissues of adult agonadal male moths, the reproductive tissues of male pupa and pharate adult *H. zea* destined to become agonadal adult male moths, and the reproductive tissues of adult males infected with Hz-2V during different life stages. Our results indicate that sterile, agonadal male moths have the ability to mate and transfer reproductive fluids containing Hz-2V to healthy female moths, without fertilizing them or altering the females' sexual receptivity to subsequent mating attempts by other healthy males. In addition, we observed Hz-2V pathology in male reproductive tissues as early as 3 days post-pupation (dpp) and Hz-2V replication in the cells of developing agonadal reproductive tissues during the pharate adult life stage.

Materials and Methods

Source of Insects and Virus

Healthy corn earworm larvae obtained from the USDA-ARS in Stoneville, MS were used to establish a laboratory colony of *H. zea*. Insects were reared on an artificial diet in environmental chambers under a L:D cycle of 16:8 hours at 28°C. Upon pupation, insects were removed from the diet, sexed, and placed into emergence chambers. After eclosion, adults were placed into mating chambers. Eggs were collected from the mating chambers, placed in hatching chambers containing artificial diet, and allowed to hatch. To prevent cannibalism, larvae were separated into individual rearing chambers when they reached late 2nd instar and maintained on artificial diet until pupation.

Hz-2V was obtained from virus-infected insects graciously supplied by Dr. John J. Hamm of the Insect Biology and Population Management Research Laboratory, USDA-ARS, Tifton, GA. The viral "waxy plugs" of agonadal female moths described by Raina *et al*. (1995 and 2000) and Hamm *et al*. (1996) were collected in phosphate-buffered saline (PBS), (136 mM NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.6 mM KCl (pH7.3)). Virus used for injection into insects was purified via sucrose gradient centrifugation (Burand and Lu 1997) and stored at -4°C.

Injection of Adults

Newly emerged healthy adult female moths were immobilized by placing them on ice and then injected with 10 ul of Hz-2V in PBS. The moths were then placed into individual mating chambers with healthy male moths and allowed to mate. Eggs were collected and reared as stated previously for the colony. Upon emergence, progeny

female moths were examined for a viral "waxy plug" indicating agonadal pathology, whereas progeny male moths required dissection and examination of the reproductive tissues to observe agonadal pathology.

Injection of Larvae, Pupae and Prepupae

Insects were immobilized by placing them on ice, and then injected with 5 ul of Hz-2V in PBS. The site of injection in pupae was sealed with melted paraffin wax. After injection, 4^{th}, 5^{th}, and 6^{th} instar larvae were placed on artificial diet and reared to the adult stage as described above. Prepupae and pupae were placed into eclosion chambers until emergence. Upon emergence as adult male moths, the insects were dissected and reproductive tissues were examined for Hz-2V pathology.

Preparation of Tissues for Electron Microscopy

Reproductive tissues to be examined by electron microscopy were dissected in PBS and fixed for at least 20 minutes in a mixture of 2% glutaraldehyde and 4% paraformaldehyde in PBS. Tissues were then transferred to fresh fixative for 2½-3 hours, washed twice with PBS at 4°C, and placed in 1% osmium tetroxide in PBS for 2 hours at room temperature. The fixed tissues were again washed twice in PBS, and subsequently embedded, thin sectioned, and stained as per Burand and Lu (1997).

Results

Pathology of Male Reproductive Tissues in Agonadal F_1 Adult Insects Arising from Hz-2V Infected Parent Females

The reproductive tissues of several hundred agonadal male moths arising from eggs laid by females that were infected with Hz-2V at the time of emergence were dissected and examined for Hz-2V pathology. Compared to the reproductive system of healthy male moths (Fig. 1A), the reproductive systems of agonadal male moths were grossly malformed (Fig. 1B). The insects lacked accessory glands, and the testes were either absent or severely underdeveloped. When present, the testes were sometimes found attached to the tips of a translucent, Y-shaped structure. In one insect, the two malformed testes were observed fused together at one tip of the Y-shaped structure. Malformed tissues composing the arms of the Y-shaped structure appeared to be grossly malformed seminal vesicles and vasa deferentia, whereas two translucent, bulb-shaped structures forming the base of the Y-shaped structure were possibly the grossly malformed ductus ejaculatorius duplex adjoined to the primary segment of the ductus ejaculatorius simplex, or primary simplex. The caudal end of the malformed primary simplex was connected with the cuticular segment of the simplex, or cuticular simplex, which was comprised of an inner cuticular duct surrounded by an outer muscular, cuticular tube. This cuticular simplex was shortened to about two-thirds of the length of the segment in a healthy male moth, and the twisted portion of the cuticular simplex, where the frenum of the spermatophore is formed, appeared only as a sharp bend in the duct near the anterior end of the segment. At the caudal end of the cuticular simplex, the inner duct adjoined the caudal end of the endophallus, whereas the outer sheath connected with the cephalad end of the aedeagus. The cephalad end of endophallus was connected to the caudal end of the

aedeagus. Tissues of the cuticular simplex, the endophallus, and aedeagus appeared to be normal, but the primary simplex and the inner duct of the cuticular simplex contained a white, viscous material, and a white, viscous, viral plug was sometimes found at the secondary gonopore, which is the opening at the caudal end of the aedeagus where the everting endophallus emerges from the caudal end of the aedeagus.

Pathology of Adult Male Reproductive Tissues Infected with Hz-2V During Different Life Stages

H. zea remained in the larval stage for approximately 2 weeks at 27°C, and took an average of 12 more days from pupation to eclosion. In order to further understand the effects of Hz-2V infection on the development of adult male reproductive tissues, we infected insects during different life stages. The reproductive tissues of adult males infected with Hz-2V during late 4^{th}, 5^{th}, and 6^{th} larval instars, and during prepupal and pupal stages exhibited various degrees of tissue malformation. Examining the reproductive tissues of these adult males injected with Hz-2V prior to the adult life stage enabled us to correlate the specific reproductive tissues that became malformed into the Y-shaped structure in agonadal adults to the corresponding tissues in healthy adults.

Whereas the reproductive tissues of adult male moths infected as 4^{th} instar males resembled those typical of F_1 agonadal males, virus infection of pupae resulted in males with normal appearing reproductive systems. Tissues from virus infected male prepupae were only slightly malformed with a noticeable reduction in the length of the accessory

glands and the primary simplex. Infection of 5th and 6th instar larvae resulted in more obvious degrees of malformation, and was most useful in determining which reproductive tissues were completely absent and which were grossly malformed in agonadal adult males.

In all 15 adult males infected with Hz-2V as late 6th instar larvae, the reproductive tissues exhibited the same moderate malformations (Fig. 2A). The accessory glands were shortened to less than one-tenth of the length of the glands in normal moths, whereas the testes were fused and appeared to be normal except in one individual, which had one severely underdeveloped testis fused to a fully developed testis. Testes were attached to apparently normal seminal vesicles, but the vasa deferentia were slightly enlarged in diameter, and the duplex tissues were slightly reduced in length compared to the tissues in normal moths. Also, the primary simplex was shortened by at least one-half of the length of the segment in normal males, with most of the reduction in length of the infected primary simplex apparently in the second secretory area of the duct. The cuticular simplex (including the twisted area where the frenum of the spermatophore is formed), the aedeagus, and the endophallus (inverted within the aedeagus) of these infected insects appeared normal.

Reproductive tracts of all 10 adult males infected as late 5th instar larvae were grossly malformed (Fig. 2B). The accessory glands appeared as a stub of malformed tissue emanating from the cephalad end of a short, swollen duct, which was the grossly malformed duplex. Whereas the fused testes seemed normally developed, they appeared to be only loosely attached to abnormally short seminal vesicles. At the caudal end of the seminal vesicles, the ducts narrowed into the enlarged vasa deferentia, which adjoined the

malformed duplex. The reproductive tract at the caudal end of the abnormal duplex merged with the grossly malformed primary simplex, the two structures forming a short, swollen, spherical duct, which then narrowed before connecting with the cuticular simplex. Though only appearing slightly malformed, the cuticular simplex was often reduced in length to about one-half that of the segment in healthy moths, and as in typical agonadal moths, the normally twisted portion of the cuticular simplex appeared as a sharp bend in the tract. The aedeagus and the inverted endophallus within appeared to be normal and functional.

Pathology of Reproductive Tissues of Different Life Stages of Insects Destined to Become Agonadal Adult Males

By 3 dpp (3 days after the larval exuviae was shed, exposing the pupa and its unsclerotized cuticle), definite differences were observed in the male reproductive tract of healthy insects and those of Hz-2V infected, agonadal insects, and these differences were even more pronounced in 4 dpp insects. Immature, diminutive reproductive tracts of healthy 4 dpp insects exhibited well-developed, fused testes attached to small seminal vesicles (Fig. 3A). The seminal vesicles narrowed caudally before adjoining the vasa deferentia, which connected with the duplex at about the midpoint of the length of the duplex. Accessory glands were approximately 2.5 mm in length and emanated from the anterior end of the duplex, which was about 2 mm long. From the duplex, the primary simplex continued for approximately 9 mm in length, before connecting to the cuticular simplex, which was close to 4.5 mm long. There was a sharp bend in the cuticular simplex just caudal to where the cuticular simplex adjoined the primary simplex. This bend is where the twisted portion of the cuticular simplex normally develops. At the

caudal end of the cuticular simplex in healthy insects, the outer sheath joined the cephalad end of the aedeagus, and the inner duct adjoined the caudal end of the endophallus, whereas the cephalad end of the endophallus connected to the caudal end of the aedeagus.

Infected, immature 4 dpp insects destined to become agonadal adults had diminutive reproductive tracts with the structure typically observed in agonadal adults (Fig. 3B). Testes were severely underdeveloped and not fused together, and the accessory glands were absent. Seminal vesicles and vasa deferentia were enlarged in diameter and malformed into a pair of tapered ducts that merged into the cephalad end of the malformed duplex, and together with the malformed duplex and primary simplex, formed a Y-shaped structure. The duplex and the primary simplex were reduced to short ducts approximately 0.8 mm and 0.5 mm long, respectively, and the cuticular simplex was reduced to less than 3.5 mm in length. There was no obvious bend in the cuticular simplex to indicate the region that becomes twisted and forms the frenum of the spermatophore in healthy male *H. zea*. The aedeagus and the endophallus within appeared to be normal. These observations show Hz-2V infection had already caused malformation in these developing male reproductive tissues prior to 4 dpp.

Ultrastructure/Hz-2V Replication

Since malformation of male reproductive tissues was seen in Hz-2V infected insects prior to 4 dpp, we examined tissues from infected insects at ages 4 to 10 dpp via electron microscopy for evidence of virus replication. Hz-2V virus particles were not observed in any of the tissues taken from insects prior to 7 dpp. In insects at 7 and 10

dpp, virus particles were observed in tissues from the grossly malformed primary simplex, which appeared as a small bulb-shaped structure. Whereas only a few cells in these tissues showed signs of virus replication at 7 dpp, high numbers of infected cells containing virus particles were visible in the tissues of 10 dpp insects. Virus-infected cells in these tissues typically were more electron dense than uninfected cells, and the nuclei of infected cells were much larger and closer to spherical in shape than those of uninfected cells (Fig. 4). Large quantities of rod-shaped, enveloped virus particles were found organized into arrays dispersed throughout these infected nuclei (Fig. 4 insert). Virus particles were also found in the lumen (Fig. 4), in intracellular spaces (Fig. 5), and in membrane-bound vesicles in the lumen of the malformed primary simplex (Fig. 6). These vesicles sometimes appeared to be nuclei that had been released from infected, lysed cells and often looked to be in various stages of deterioration. No virus particles were ever observed in cells of the cuticular simplex.

Discussion

The fertilization of the female corn earworm moth during copulation involves the transference of a spermatophore and other secretions of the male reproductive tract (Callahan *et al.*1958, 1963), including anti-calling factors and the PSP described by Raina *et al.* (1994) and Kingan *et al.* (1995). At the initiation of copulation, the male's endophallus everts into the cervix bursa of the bursa copulatrix in the female moth. Contractions of the primary and cuticular simplexes move the secretions of the male reproductive tract through the male's endophallus and into the cervix bursa of the female. The primary simplex secretes the spermatophore precursor, which, as it is transferred to the female, becomes filled with a mixture of sperm from the testes and secretions of the male's duplex and accessory glands. After the spermatophore has been completely transferred to the female moth, the male withdraws the endophallus. Male accessory gland secretions, delivered to the female with the spermatophore, stimulate muscular contractions in the bursa copulatrix to force the sperm out of the spermatophore and into the seminal duct of the female.

Beginning during the second scotophase after emergence as adults, the female moths release sex pheromones and begin a "calling" behavior, rapidly vibrating the wings while extruding the ovipositor, to attract a mate. Kingan *et al.* (1993, 1995) and Raina *et al.* (1994) reported that the transfer of anti-calling factors, including the spermatophore and associated secretions produced in the primary simplex, into the corpus bursa of a female moth triggers a cessation of calling and the loss sexual receptivity of the female for the rest of that scotophase. In addition, the male accessory glands secrete a PSP, which is transmitted within the spermatophore to the female moth during copulation,

resulting in a severe drop in the pheromone production of the female moth (Raina *et al.* 1994; Kingan *et al.* 1995). The transference of the spermatophore and the PSP causes the female to switch from displaying sexually receptive or 'virgin' behavior, to displaying unreceptive or 'mated' behavior (Raina *et al.* 1994; Kingan *et al.* 1995), ultimately resulting in the female mating no more than once during each of the second and subsequent scotophases.

Kingan *et al.* (1993) reported that surgically altered healthy males, in which the duplexes, accessory glands, vasa deferentia, seminal vesicles, and testes were removed, were still able to mate and transfer the spermatophore and secretions of the simplex into the cervix bursa of the female moth. This indicates that only the lower portion of the male reproductive tract, the simplex, aedeagus, and endophallus, are essential for the successful initiation of copulation and transfer of reproductive fluids into the female moth.

Our examination of the reproductive tracts of agonadal male insects and males infected with Hz-2V at various life stages shows that whereas the accessory glands are absent and the seminal vesicles, vasa deferentia, duplex, and primary simplex are grossly malformed into a Y-shaped structure in agonadal male *H. zea*, the slightly malformed cuticular simplex appears to be functional, and the aedeagus and endophallus appear to be normal and functional. The condition of these tissues indicates that although much of the reproductive system in agonadal adult males is grossly malformed, the reproductive tissues essential for successful initiation of copulation and transmission of reproductive fluids may be functional.

Hz-2V replication was found in the Y-shaped structure in a few infected cells of the malformed primary simplex in agonadal insects as early as 7 dpp. It seems likely that replication begins prior to 7 dpp since malformation of these tissues was observed as early as 3 dpp. It is possible that the virus replication begins early on in the development of the reproductive tissues, and that the replication process affects the development of the reproductive system without overt signs of virus replication. It is also possible that early on in development, some viral encoded factor(s) is produced that alters the development of the reproductive system, reprogramming it to develop into tissues that are more favorable for virus replication.

By 10 dpp, the proportion of infected cells in the primary simplex had increased dramatically, and the quantity of virus particles in these tissues had increased exponentially. Small quantities of white, viscous material, as described within the simplex of adult agonadal males, began to be visible in the malformed primary simplex of 10 dpp males. We suggest that this white, viscous material contains concentrated virus particles and is similar to the white, buttery mass in the bursa copulatrix of agonadal female moths described by Raina *et al.* (2000) as containing "atypical" viral occlusion bodies.

These observations indicate that sterile, agonadal male moths are capable of mating with and infecting healthy female moths. We suggest that during these matings, muscular contractions in the cuticular simplex draw reproductive fluid containing Hz-2V from within the lumen of the malformed reproductive tract and force this virus-contaminated fluid through the endophallus, into the cervix bursa of the female. Transfer of the virus with this fluid could result in Hz-2V contamination or infection of the female

moth without her becoming fertilized and without altering her sexual receptivity, since the male's reproductive tissues responsible for secreting the anti-calling factors and the pheromonostatic peptide are either absent or grossly malformed. The newly contaminated female may then mate with a healthy male during that same scotophase or during a later scotophase and receive sperm for her developing eggs, many of which could also become infected with Hz-2V.

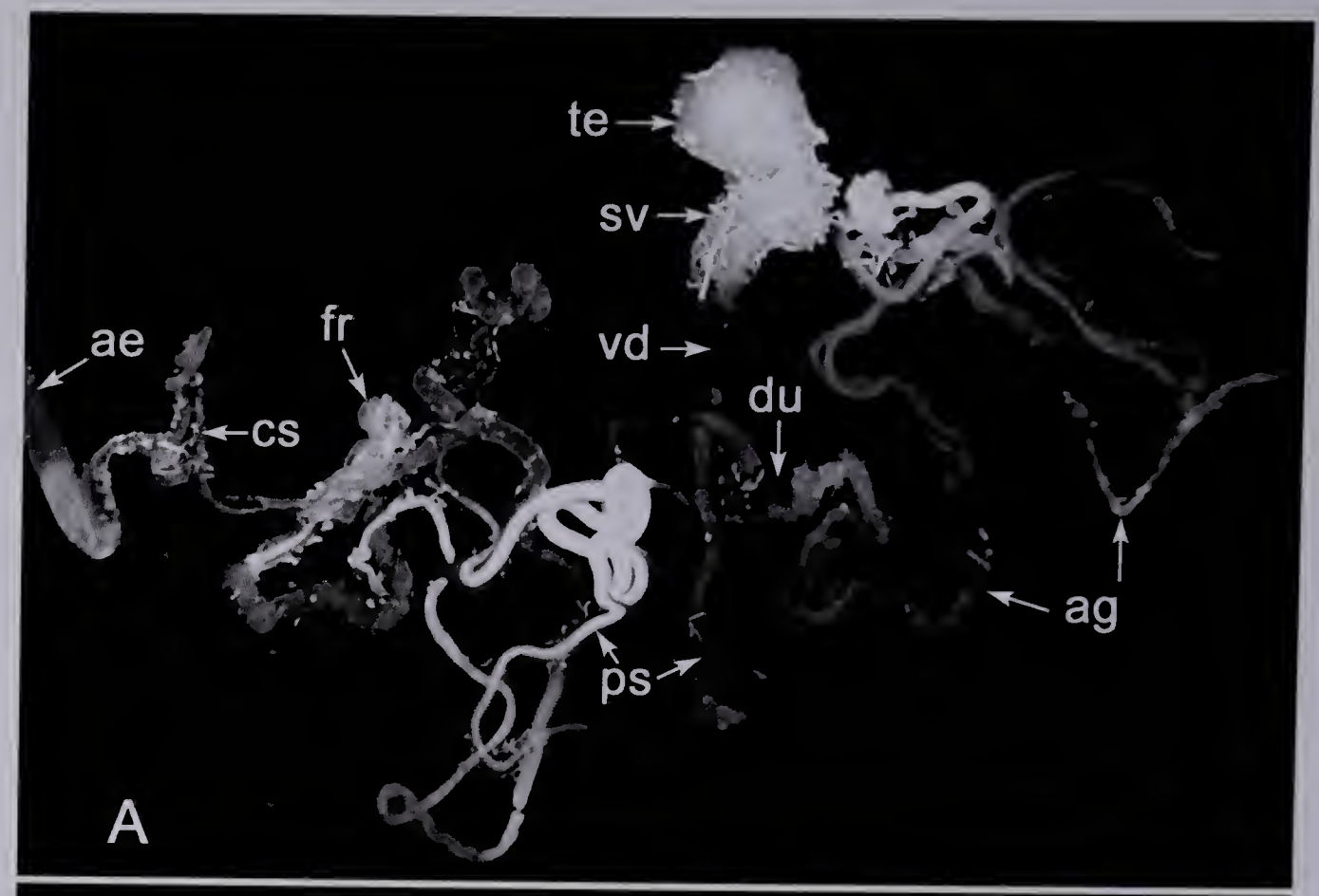
Our results and the results of mating experiments conducted by Hamm *et al.* (1996) indicate that infected male moths are capable of mating with and transmitting Hz-2V to healthy females, and that these newly contaminated females are able to transmit the virus to developing eggs. In the Hamm *et al.* experiments, infected progeny moths arose from eggs laid by healthy females that had been mass mated with Stoneville males only during one scotophase. No infected progeny were recovered from eggs laid by healthy females mated individually with Stoneville males. Since Hamm *et al.* (1996) could not distinguish between asymptomatic males and uninfected males, it is not known whether any males used in these experiments were fertile, asymptomatic carriers of Hz-2V. It is possible that any asymptomatic male moths present in the mass-mating experiment could have transmitted the virus with reproductive fluids to the healthy females during copulation. Although unlikely, it is also possible that none of the male moths mated with healthy females in the individual chambers were fertile, infected, asymptomatic carriers.

Based on our observations of pathology in Hz-2V infected, agonadal males, another more likely explanation is that the results observed by Hamm *et al.* (1996) may have arisen from matings involving virus-infected, agonadal male moths. Agonadal, sterile males seem to be physically able to mate with healthy females, transmitting virus

without fertilizing the female moths or altering their sexual receptivity. Matings between agonadal males and healthy females in the individual mating chambers would result in Hz-2V contamination or infection of the females, but no viable progeny. However, in the mass-mating experiment conducted by Hamm *et al.*, after mating with virus-infected, agonadal males, the newly contaminated females would likely remain receptive to additional matings, and mate with healthy males during the same scotophase, resulting in infected, viable progeny.

The data presented here indicate that the evolution of Hz-2V in *H. zea* seems to have resulted in the ability of the virus to alter the development of the reproductive system in male *H. zea* in order to achieve a high level of virus replication, as well as increase the efficiency of its transmission by coupling virus transmission with copulation. Agonadal males appear to be able to mate with and transmit Hz-2V to healthy female moths. Normally newly mated females would not mate again until the next scotophase. However, infection with Hz-2V likely disrupts the production of pheromonostatic and anti-calling factors in the agonadal male moth. Therefore, females that mate with agonadal males could, during the same scotophase, become infected and remain receptive to additional matings with healthy males and receive sperm to fertilize their eggs. Since any subsequent matings with contaminated females could potentially result in the contamination of healthy males, the disruption of healthy female mating behavior by Hz-2V may enhance the speed and efficiency of virus transmission and favor virus transmission in the population of insects.

Figure 2.1. Healthy and agonadal reproductive systems of the adult male *H. zea*. (A) Healthy male, accessory glands (ag), testes (te), seminal vesicles (sv), vasa deferentia (vd), duplex (du), primary simplex (ps), cuticular simplex (cs), twisted area of the cuticular simplex where the frenum of the spermatophore is formed (fr), and the aedeagus (ae). (B) Agonadal male exhibiting grossly malformed reproductive system lacking testes and accessory glands, but exhibiting other tissues corresponding to the same tissues of healthy male.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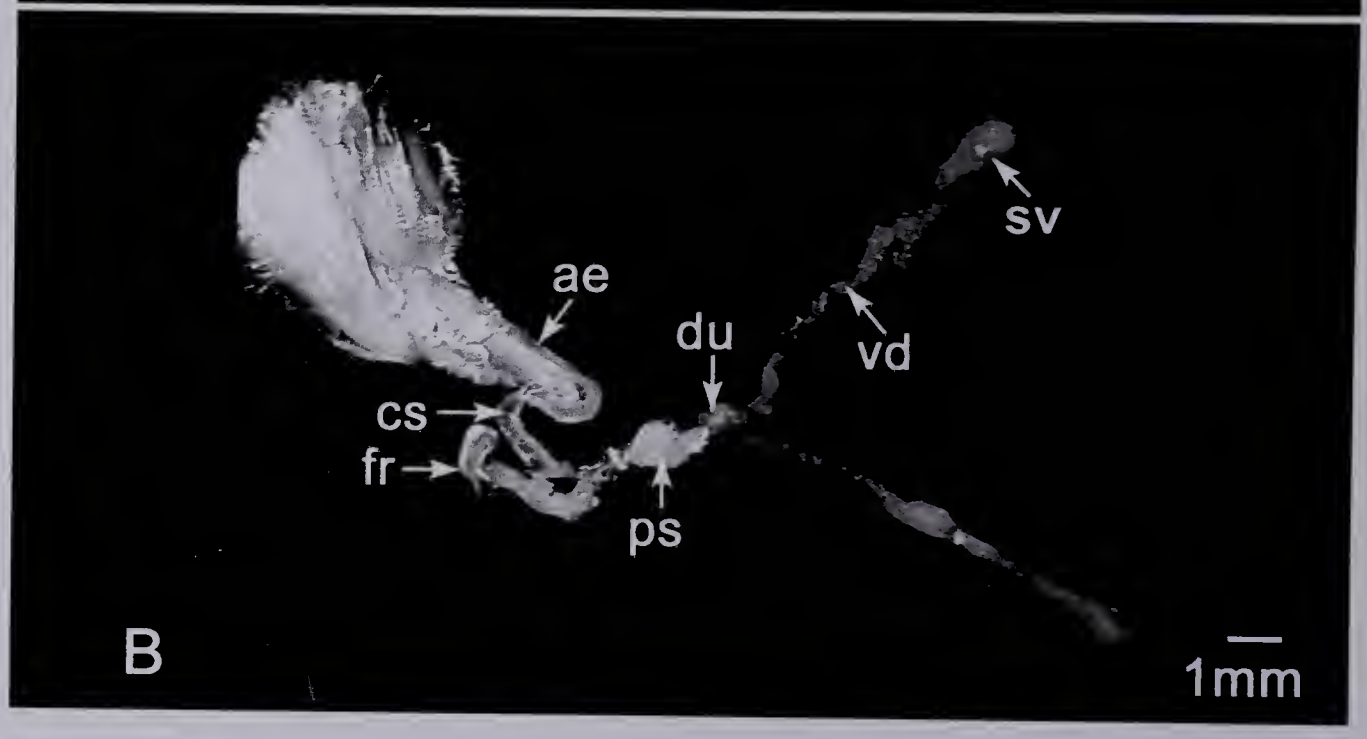
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Figure 2.2. Malformed reproductive systems of adult male *H. zea* infected with Hz-2V during the late 6^{th} (A) and 5^{th} (B) instars. Accessory glands (ag), testes (te), seminal vesicles (sv), vasa deferentia (vd), duplex (du), primary simplex (ps), cuticular simplex (cs), twisted area of the cuticular simplex where the frenum of the spermatophore is formed (fr), and the aedeagus (ae). Note the vast difference in development of the primary simplex (ps), duplex (du), and accessory glands (ag).

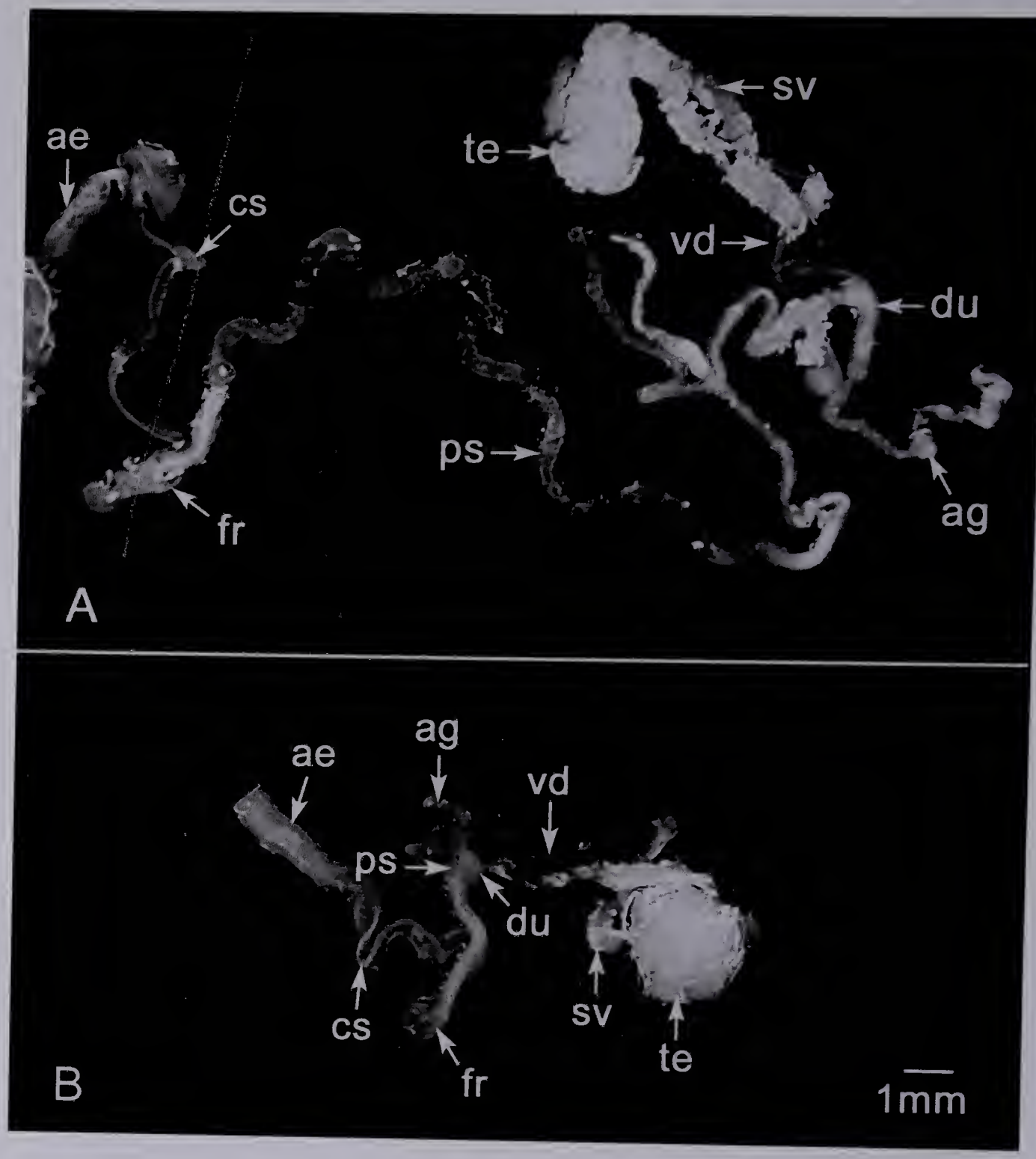
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Figure 2.3. Healthy and agonadal reproductive systems of 4 dpp male *H. zea.* (A) Healthy 4 dpp male, accessory glands (ag), testes (te), seminal vesicles (sv), vasa deferentia (vd), duplex (du), primary simplex (ps), cuticular simplex (cs), twisted area of the cuticular simplex where the frenum of the spermatophore is formed (fr), and the aedeagus (ae). (B) Agonadal 4 dpp male exhibiting a malformed reproductive system with underdeveloped testes (te), which have not fused together as in healthy males and are attached to the tips of the malformed seminal vesicles (sv).

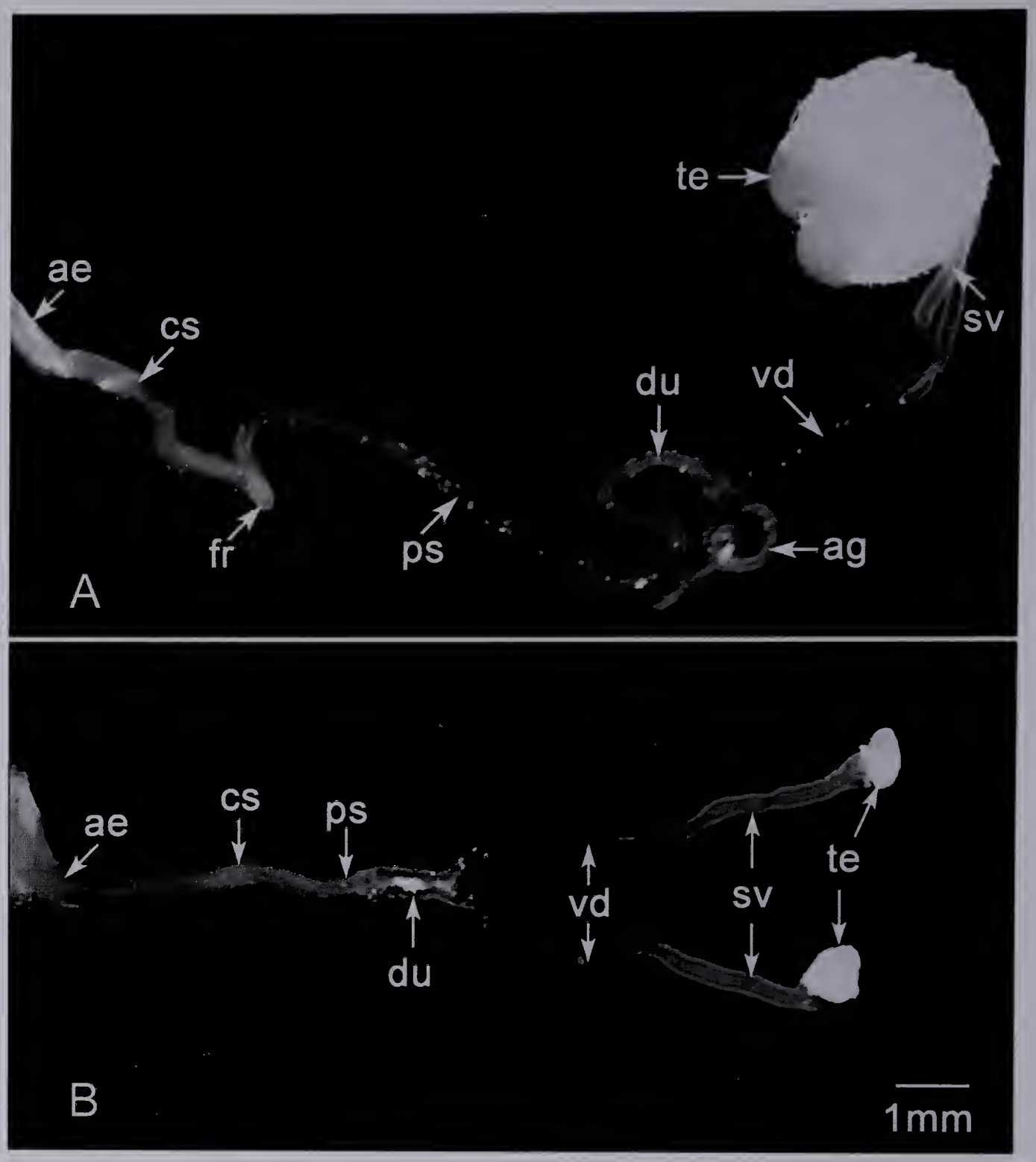
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Figure 2.4. Electron micrograph of the malformed primary simplex tissue of a 10 dpp male pharate adult *H. zea* infected with Hz-2V. Rod-shaped virus particles (V) are visible in the lumen (L) of the simplex and in nuclei (Ni) of infected cells. The virus particles in infected nuclei are organized into arrays scattered throughout the nucleus (insert). Infected cells were more electron-dense and therefore stained more darkly than uninfected cells.

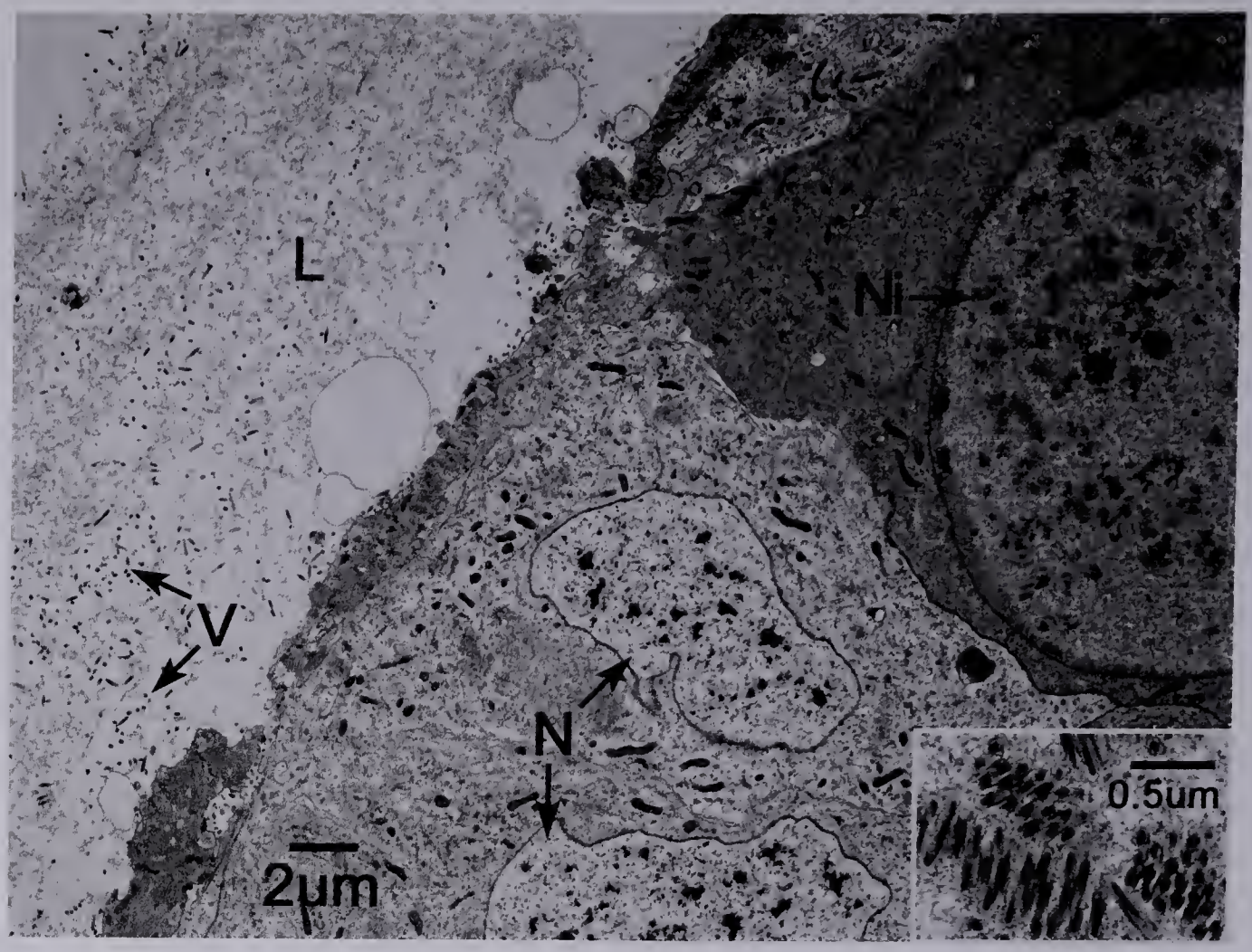

L
Ni
V
N
2µm
0.5um

Figure 2.5. Electron micrograph of intracellular virus particles in primary simplex tissue of a 10 dpp pharate adult infected with Hz-2V. Intercellular virus particles (V), likely released from a lysed cell, border an infected cell with a virus-filled nucleus (N) that appears close to lysing.

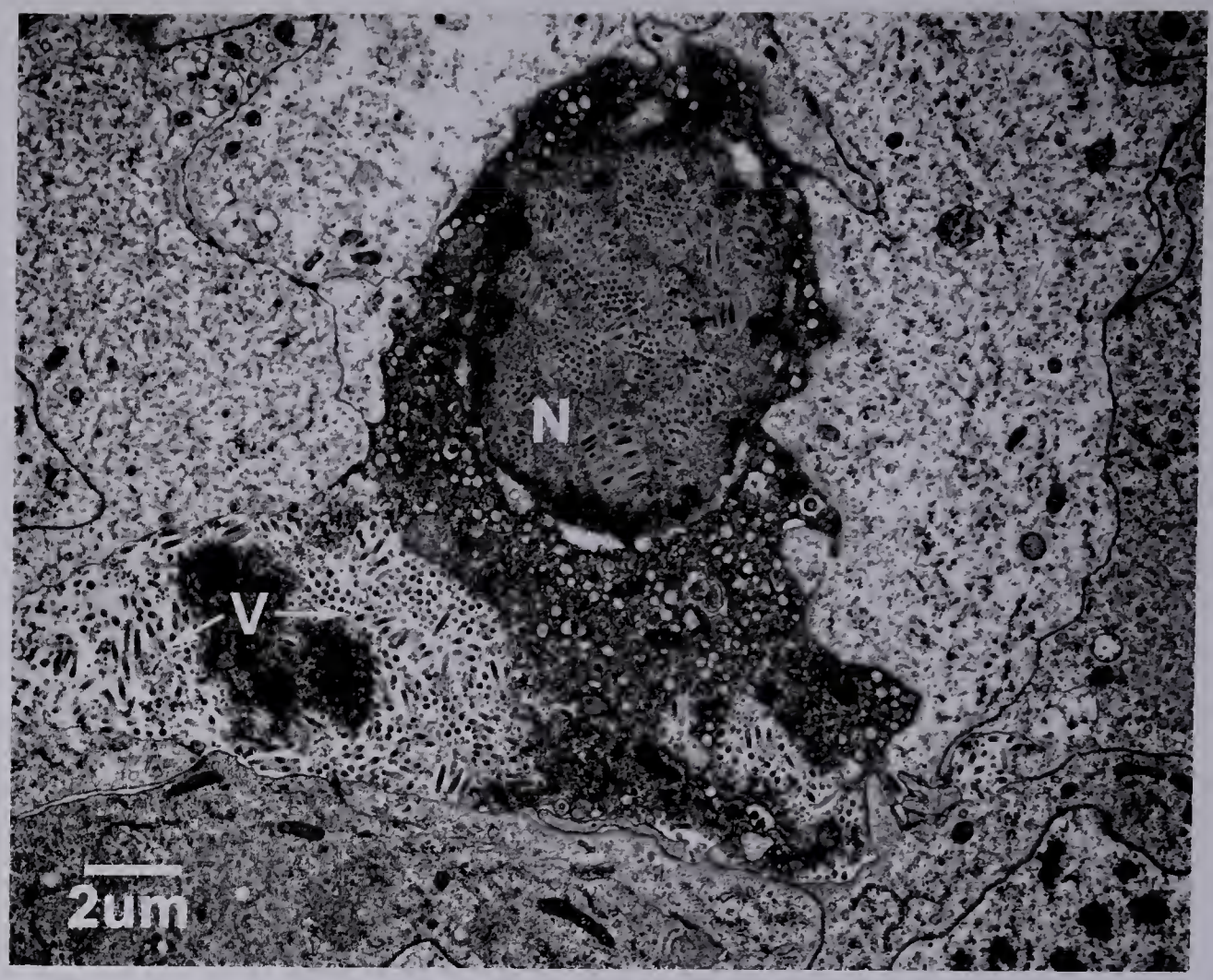
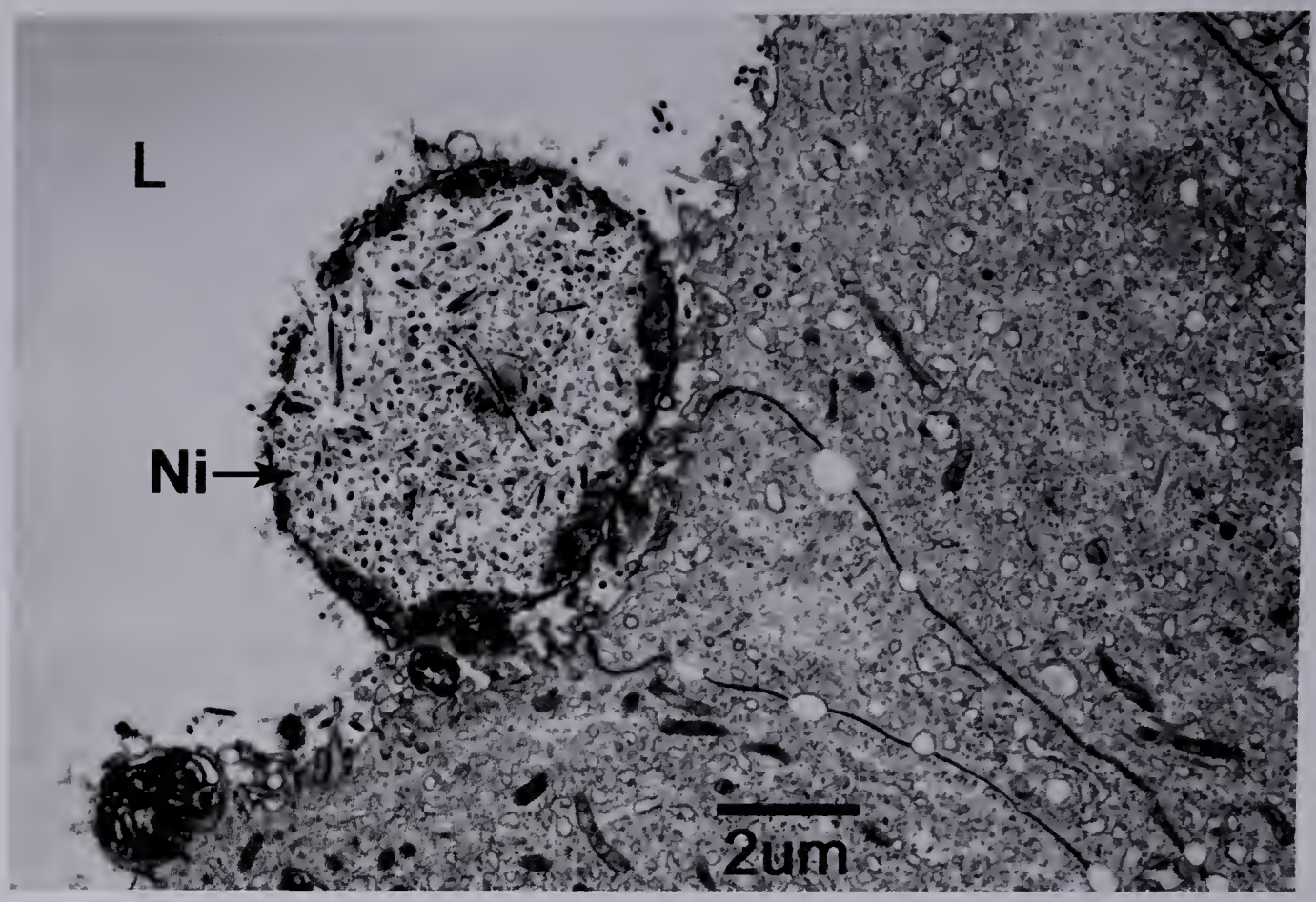

N
V
2um

Figure 2.6. Electron micrograph of a virus-filled vesicle (Ni) in the lumen (L) of a malformed primary simplex of a 10 dpp male pharate adult infected with Hz-2V. The membrane of the vesicle has deteriorated in some areas, releasing virus particles into the lumen.


L
Ni
2um

CHAPTER 3

HZ-2V INFECTION IN FEMALE *HELICOVERPA ZEA*

Introduction

Although described previously by Raina and Adams (1995) and Hamm *et al.* (1996), the grossly malformed reproductive tissues in infected female corn earworm moths, *Helicoverpa zea*, have not been definitively identified with analogous reproductive tissues found in healthy moths. Identification of these malformed tissues is essential in order to compare the ultrastructure of the reproductive tissues in agonadal and healthy female moths to determine the effects of virus infection on tissue development and differentiation. We have found that infecting female *H. zea* during different life stages results in a progression in the malformation of the reproductive tissues. Following this progression has allowed us to determine which malformed tissues are analogous to the reproductive tissues in healthy female corn earworm moths.

Hz-2V induced malformation of the reproductive tissues in female corn earworm moths results in the development of a large, Y-shaped reproductive structure adjoining a smaller C-shaped structure (Hamm *et al.* 1996). Malformation of these tissues was observed as early as 3 dpp. An external symptom of the agonadal condition in adult female moths is the presence of a viscous, white "waxy plug" coalesced about the pair of reproductive openings at the tip of the abdomen. This "waxy plug" has been described as containing large quantities of vesicles filled with virus particles (Hamm *et al.* 1996).

Productive virus replication has been detected primarily in epithelial cells that make up the Y-shaped structure (Hamm *et al.* 1996), but whereas Hz-2V replication has been studied *in vitro* (Burand and Lu 1997; Raina *et al.* 2000; Lu and Burand 2001), little

is known about the replication of the virus *in vivo* in the corn earworm moth. In addition, the origin and role that the virus-filled "waxy plug" plays in the biology of the virus have not yet been determined. It appears that the virus expends a considerable amount of energy in the production of this virus-filled plug, suggesting that the "waxy plug" may play an important role in the ecology and transmission of Hz-2V.

Ultrastructural examination of the tissues comprising the Y-shaped structure revealed that the cells in these Hz-2V infected tissues had proliferated, resulting in an increased number of cells capable of supporting virus replication. Furthermore, virus infection in agonadal moths prevents the development of the membranous lining found in the oviducts in healthy moths. The absence of this lining and the proliferation of cells in the malformed reproductive tissues of agonadal moths ultimately lead to the production of a large amount of virus in these tissues. Two to three days prior to eclosion, large numbers of virus particles were found accumulating in the malformed cervix bursa of the bursa copulatrix of agonadal pharate moths. Immediately after eclosion, a "waxy plug" was found covering the vulva. This suggests that as the agonadal female moth emerges, the virus exudes out of the vulva and ultimately forms the viral "waxy plug".

Materials and Methods

Source of Insects and Virus

Uninfected corn earworm larvae obtained from the USDA-ARS in Stoneville, MS were used to start a laboratory colony of healthy *H. zea*. Insects were reared on artificial diet in environmental chambers under a L:D cycle of 16:8 hours at 28°C. Upon pupation, insects were removed from the diet, sexed, and placed into emergence chambers. After eclosion, adult moths were placed into mating chambers. Eggs were collected from the mating chambers, placed into hatching chambers containing artificial diet, and allowed to hatch. To prevent cannibalism, when larvae reached late 2nd instar, they were separated into individual rearing chambers containing artificial diet and reared individually until pupation.

Hz-2V-infected insects were graciously supplied by John J. Hamm of the Insect Biology and Population Management Research Laboratory, USDA-ARS, Tifton, GA. The viral "waxy plugs" from agonadal female moths were collected in phosphate-buffered saline (PBS: 136 mM NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.6 mM KCl; pH7.3). Virus used for injection into insects was purified via sucrose gradient centrifugation (Burand and Lu 1997) and stored at 4°C.

Injection of Adults

Newly emerged, healthy adult female moths were placed on ice to immobilize them and then injected with 10 ul of Hz-2V in PBS. The moths were placed into individual mating chambers with healthy male moths and allowed to mate. Eggs were

collected and reared as stated previously for the colony. Upon emergence, progeny adult female moths were examined for agonadal pathology.

Injection of Larvae and Prepupae

Healthy insects were placed on ice to immobilize them and then injected with 5 ul of Hz-2V in PBS. The site of injection in pupae was sealed with melted paraffin wax. After injecting 4^{th}, 5^{th}, and 6^{th} instar larvae, they were placed on artificial diet to be reared to the adult stage as described above for the colony. Prepupae and pupae were placed into eclosion chambers until emergence. Upon emergence as adult moths, the insects were dissected and reproductive tissues were examined for Hz-2V pathology.

Preparation of Tissues for Electron Microscopy

Reproductive tissues to be examined by electron microscopy were dissected in PBS and fixed for at least 20 minutes in a mixture of 2% glutaraldehyde and 4% paraformaldehyde in PBS. Tissues were then transferred to fresh fixative and allowed to fix for 2.5-3 hours at $4^{o}C$. Tissues were washed twice with PBS at $4^{o}C$ and then placed in 1% osmium tetroxide in PBS at room temperature for 2 hours. After washing twice in PBS, fixed tissues were embedded, thin-sectioned, and stained as per Burand and Lu (1997).

Results

Pathology of Adult Female Reproductive Tissues from Insects Infected with Hz-2V During Different Life Stages

The reproductive tissues of healthy female corn earworm moths were extensive and filled the abdominal cavity (Fig. 1A). Terms used here to describe these tissues are those used by Callahan (1958). The most obvious tissue was a pair of ovaries, each comprised of four very long ovarioles containing eggs in successive stages of development. In each ovary, the four ovarioles merged at the caudal end to form the calyx, which then tapered slightly before connecting to the lateral oviducts. The lateral oviducts were very short and merged together to become the common oviduct, which terminated at the caudal reproductive opening, the oviporus. The oviducts in healthy females were a constant diameter, and all of the ovary and oviduct tissues were transparent.

Arising from the caudal region of the common oviduct via separate ducts were the spermatheca, the accessory glands, and the seminal duct, which linked the common oviduct to the bursa copulatrix. The bursa copulatrix was comprised of two lobes, the larger of which was the corkscrew-shaped cervix bursa, the cephalad end of which was connected to the seminal duct. During copulation, the cervix bursa receives the endophallus of the male moth, whereas the smaller, flattened, muscular lobe of the bursa copulatrix, the corpus bursa, receives the bulbous head of the spermatophore (Callahan 1963). The two lobes of the bursa copulatrix merged at the caudal end and tapered sharply to become the ductus bursa, which connected the bursa copulatrix to the anterior reproductive opening, the vulva.

In agonadal female moths arising from eggs laid by Hz-2V-injected females, the structure of the reproductive tissues was far different from that found in healthy insects (Fig. 1D). The entire reproductive structure was malformed into a large Y-shaped structure connected at the caudal end to a smaller, swollen C-shaped structure. The Y-shaped structure was translucent white in color, and was much greater in diameter than the oviducts and ovarioles of uninfected insects. The ovarioles, spermatheca, accessory glands, seminal duct, and bursa copulatrix were absent. At its caudal end, the large Y-shaped structure terminated at the oviporus. The C-shaped structure merged at its cephalad end with the caudal region of the Y-shaped structure, and a small duct connected the caudal region of the C-shaped structure to the vulva. All of the tissues contained a white, viscous substance that was most concentrated in the swollen C-shaped structure during the latter days prior to the eclosion of the adult moth.

Injecting female insects with Hz-2V during different life stages and rearing these insects to adults resulted in a pattern of progressive malformation of reproductive tissues that increased as the insects were infected earlier in their development (Table 1). Whereas virus infection of pupae resulted in adult females with apparently normal reproductive tissues, the infection of prepupae produced adults with slightly malformed common and lateral oviducts, which were significantly enlarged in diameter compared to oviducts of uninfected moths (data not shown). Infection of 5^{th} and 6^{th} instar larvae with Hz-2V resulted in an increase in the amount of malformation in adult reproductive tissues. In adults infected as 4^{th} instars, malformation of these tissues closely resembled that observed in agonadal moths that arose from eggs laid by females infected with Hz-2V as adults (compare healthy, Fig 1A, and infected, Fig. 1D). The pattern observed in the

malformation of these reproductive tissues served to help identify the tissues that make up the Y-shaped structure in agonadal adults.

Although reproductive tissues of moths infected with Hz-2V as pupae and prepupae looked almost normal, the reproductive system of adult females infected as late 6^{th} instar larvae were obviously malformed (Fig. 1B). Some of the ovarioles were grossly malformed, yet other ovarioles in the same insects appeared to be fully developed and contained eggs in successive stages of development. The lateral oviducts of these insects were enlarged in diameter and more than doubled in length compared to these tissues in uninfected moths. Whereas the common oviduct in these insects was enlarged to about three times its normal diameter at the cephalad end, the oviduct narrowed sharply below the area where the oviduct joined the cervix bursa. The seminal duct, which in healthy moths is a narrow duct connecting the cervix bursa to the common oviduct, was absent in agonadal moths infected as 6^{th} instar larvae. This resulted in the common oviduct being joined directly to the cervix bursa, which in these insects had become malformed into a swollen duct. Although the corpus bursa was present, it was reduced in size and grossly malformed. The spermatheca in these infected insects was absent, but some tissues were present that appeared to be a short web of malformed accessory glands and gland reservoirs.

Adult females infected as 5^{th} instar larvae exhibited grossly malformed reproductive systems (Fig. 1C). All ovarioles were malformed, leaving just short stubs of ovariole tissue emanating from the tips and sometimes the lower regions of the lateral oviducts. Remnants of ovarioles containing several severely underdeveloped oocytes were sometimes observed. The lateral oviducts in these insects were translucent and

grossly malformed into enlarged ducts longer than the common oviduct. The common oviduct was also translucent and was enlarged to more than four times its normal diameter at the junction with the lateral oviducts, but narrowed sharply at the point where it joined with the cervix bursa, which appeared as a swollen, white, sinuous duct. The seminal duct was absent, and the shrunken, malformed corpus bursa of the bursa copulatrix projected from the grossly malformed cervix bursa. The spermatheca was also absent, and the accessory glands were reduced to a tiny Y-shaped duct emanating from the caudal end of the common oviduct.

Pathology of Reproductive Tissues in Different Life Stages of Insects Destined to Become Agonadal

The structures of the healthy female adult reproductive system were already apparent in insects at 3 dpp (not shown). At 4 dpp these tissues were more completely formed (Fig. 2A), and were composed of eight long, narrow, partially developed ovarioles which arose from finger-like pedicels. These pedicels merged at the caudal end to form the pair of calyxes that then connected to the lateral oviducts, which at this stage were only slightly over 0.5 mm long and 0.2 mm in diameter. The lateral oviducts merged caudally to form the common oviduct, which was approximately 1 mm long and about the same diameter as the lateral oviducts. The common oviduct terminated at the oviporus, the posterior external reproductive opening (not shown). Small, paired accessory gland reservoirs emanated from the caudal region of the common oviduct, and tapered into long, narrow accessory glands. Anterior to the common oviduct, the bursa copulatrix arose from the narrow ductus bursa, which widened caudally into the vulva, the anterior external abdominal opening (not shown). Whereas the corpus bursa was a

short, balloon shaped structure about 1 mm in length, the other arm of the bursa copulatrix, the cervix bursa, was twisted into a corkscrew structure approximately 4 mm in length. A narrow seminal duct connected the cephalad end of the cervix bursa to the caudal region of the common oviduct.

In contrast to the reproductive tissues of healthy 3 dpp insects, the reproductive tissues of infected 3 dpp (not shown) insects destined to become agonadal adults had already developed into malformed structures. By 4 dpp they resembled the typical structures observed in agonadal adult female moths (Fig. 2B). In these 4 dpp insects destined to become agonadal adult female moths, the ovarioles were absent or appeared as stubs of hypertrophied tissue approximately 1 mm in length attached to the tips of the grossly malformed lateral oviducts. The hypertrophied lateral oviducts were enlarged to over 5 mm in length and often more than 0.5 mm in diameter, and dominated the agonadal reproductive structure. Where the lateral oviducts merged to become the common oviduct, the oviduct was enlarged to over 1 mm in diameter, but tapered to approximately 0.25 mm where it connected to the grossly malformed cervix bursa, and continued to narrow below the cervix bursa before terminating at the oviporus. Rather than being connected to the common oviduct via a narrow seminal duct as in the normal reproductive system, the cephalad end of the malformed cervix bursa was joined directly to the common oviduct. The caudal end of the cervix bursa was connected to the vulva via a slightly malformed ductus bursa. The other arm of the bursa copulatrix, the corpus bursa, was severely reduced in size.

Ultrastructural Analysis of Hz-2V Replication

The presence of malformed reproductive tissues in virus-infected insects at 3 dpp indicates that Hz-2V had begun to influence the development of these tissues early on in their development. Based on these observations, the lateral and common oviducts and the cervix bursae of female insects at 4 dpp to 10 dpp were examined via electron microscopy for evidence of Hz-2V replication.

The tissues of healthy lateral and common oviducts varied in thickness, but were ordinarily comprised of a single layer of epithelial cells bordered on the basal side by the basement membrane, and on the apical side by a thick membranous layer (Fig. 3A). Generally, each cell contained a spherical to ovoid nucleus, which comprised about 20-30% of the cell's contents.

The membranous lining was first observed in healthy 7 dpp insects in both oviducts. The lining of the common oviduct in these insects was comprised of a thick procuticle of variable width lined by a thin epicuticle (Fig. 3A inset). The lining appeared more organized than that of the lateral oviduct, which was comprised of a thin procuticle and epicuticle. The membranous layer of the lateral oviducts in these insects became more diffuse at the cephalad end of the ducts (Fig. 3D). By 10 dpp, the membranous layer of the lateral oviduct had increased to proportions similar to that of the common oviduct, and at the time of eclosion of the adult female moth, the linings of the common and lateral oviducts were very similar in appearance. At this stage, the thick procuticle of the oviducts had diminished, whereas the epicuticle had increased by almost eight-fold (Fig. 3C).

In contrast to the single layer of epithelial cells in oviduct tissues of healthy insects, the hypertrophied tissues of the lateral and common oviducts from Hz-2V-infected, agonadal insects were comprised of unorganized masses of cells bordered on the basal side by the basement membrane (Fig. 3B). The thick, membranous layer that lined the apical side of the cells of healthy oviducts was completely absent in tissues from agonadal insects, resulting in cells being directly exposed to the lumen. These cells in malformed oviducts often formed microvilli that projected into the lumen (Fig. 3B inset). In sharp contrast, microvilli were never observed from cells of normal oviducts.

Although there was a four- to eight-fold increase in the number of cells in oviduct tissues from infected insects compared to those tissues in healthy *H. zea*, at 4 dpp most of the cells appeared normal and were without any visible signs of virus replication (Fig. 3B). Virus particles were observed in very few nuclei of cells and occasionally in the lumina in the hypertrophied lateral oviducts as early as 4 dpp and in common oviducts at 7 dpp. Infected nuclei were more numerous in the cephalad region of the malformed lateral oviducts, and the frequency of infected nuclei containing virus particles and the numbers of virus particles found in the lumina increased as the insects matured. In the lateral oviducts at 10 dpp, virus particles were observed in infected nuclei of clusters of infected cells and in the lumina (Fig. 4A). A similar trend was observed in the common oviducts, except fewer cells containing infected nuclei were found and lower numbers of virus particles were observed in the lumina.

The cytoplasm of cells containing virus particles stained more darkly and was more uniform in appearance than that of healthy cells. The nuclei of these virus-infected cells were greatly enlarged, often constituting more than 80% of the cells' contents (Fig.

4B). Clusters of arrays of enveloped virus particles, as previously described (Hamm *et al.* 1996; Burand and Lu 1997; Raina *et al.* 2000; and Lu and Burand 2001), were frequently observed in infected nuclei, and the nuclear membranes of some of these cells sometimes appeared to be absent or possibly degraded (Fig. 4A).

Numerous free virus particles were observed in the lumina of the lateral and common oviducts in 10 dpp insects (Fig. 4A). At this time, some oviduct cells containing virus particles were found to be lysing, releasing virus and cellular components into the lumen (Fig. 5A). Large membrane-bound vesicles that also contained virus particles and possibly some cellular debris were often observed in the lumina of infected oviducts. These vesicles appeared to be the remnants of infected cell nuclei and were frequently observed lysing, releasing virus particles into the lumina (Fig. 5B). In some instances these vesicles were observed at cellular junctions along the lumina (Fig. 5C). These vesicles did not appear to be the same as those described previously in the oviduct (Raina and Adams 1995), the "waxy plug" (Hamm *et al.* 1996), or the malformed bursa copulatrix of agonadal female moths (Raina *et al.* 2000).

Like the oviduct tissues, the cervix bursa of the bursa copulatrix in healthy adult female *H. zea* was also comprised of a single layer of epithelial cells bordered on the basal side by a basement membrane (Fig. 6A). However, lining the apical side of the epithelial cells was a very thick layer of procuticle. The procuticle in the cervix bursa was greater in width than any of the other reproductive tissue and appeared as a series of alternating layers of light and dark staining tissue lined by a thin epicuticle (not shown).

Although the cervix bursae in agonadal adult female *H. zea* were grossly malformed, ultrastructural examination revealed that the tissues (not shown) were similar

to that of the cervix bursae of healthy females. There was a single layer of epithelial cells bordered on the apical side by a procuticle less than one-tenth the width of that of the cervix bursae in healthy females, and an epicuticle that appeared to be of normal width. No virus-infected nuclei were observed in the cervix bursae of agonadal moths.

During the latter days of pharate adult development, the malformed cervix bursa became white and swollen, and was filled with a white, viscous material. Ultrastructural examination of infected cervix bursae at 10 dpp revealed that the white viscous material was a mass of concentrated virus particles embedded in a dark granular substance (Fig. 6B).

Examination of the cervix bursae of newly emerged agonadal adult female moths revealed that the masses of virus particles observed in the bursae of 10 dpp agonadal pharate adults had become separated into various-sized spheres comprised of virus particles intermingled with a dark-staining granular substance (Fig. 7A). These spheres were not membrane-bound and often had ragged edges where some virus particles may have been dissociating from them (Fig. 7B). Otherwise the spheres were very similar in appearance to the atypical occlusion bodies previously described in the oviduct (Raina and Adams 1995), the "waxy plug" (Hamm *et al.* 1996), and the malformed bursa copulatrix of agonadal female moths (Raina *et al.* 2000).

Discussion

The most obvious pathology in agonadal females was the gross malformation of the common and lateral oviducts resulting in a large Y-shaped structure. Productive virus replication was detected primarily in epithelial cells that make up this Y-shaped structure. Very few virus-infected nuclei were observed in the cells of the oviducts until 6 to 7 dpp. However, a few vesicles containing virus particles were found in the lumen of the lateral oviduct of one insect aged 4 dpp, suggesting that Hz-2V replication begins in cells of the cephalad region of the lateral oviducts in insects before 4 dpp. Since agonadal pathology was observed in insects as early as 3 dpp, it appears that virus activity early in the development of the reproductive tissues affects the subsequent development of these tissues without overt signs of virus replication.

By 4 dpp, the malformed oviducts of the Y-shaped structure in agonadal insects were several times larger than the tissues in normal insects and no longer consisted of a single layer of epithelial cells, but were comprised of several distinct layers of cells. The four to eight-fold increase in the number of cells observed in these infected, hypertrophied oviducts indicates that cell proliferation occurred in these tissues as a result of virus infection. Hz-2V was observed to replicate in the nuclei of these infected cells, causing the nuclei to expand to comprise greater than 80% of the cells. This culminated in the production of a large number of virus particles, which were found arranged in numerous arrays inside the expanded nuclei. Thus, the proliferation of cells in these tissues results in conditions favorable for virus replication, ultimately enabling Hz-2V to achieve very high titers in the oviducts of agonadal female moths.

Ultrastructural examination of oviduct tissues revealed that infection with Hz-2V prevents the development of the cuticular lining of the lumen in the malformed oviducts of agonadal moths. The cuticular lining of the oviducts in healthy female moths borders the apical side of the epithelial cells and forms the lumina of the ducts. The insect cuticle and the chitinous peritrophic matrix, which lines the midgut of insects, serve as barriers to infection of the insect by pathogens, and the cuticular lining of the common oviduct possibly serves the same purpose in adult insects. The lining of the lateral oviduct is very similar to that of the common oviduct and may also function as a barrier to infection. The absence of this lining in agonadal insects exposes the epithelial cells of the oviduct to the lumen and likely enables virus from infected cells to readily enter into the lumen and then infect healthy cells along the length of the oviducts, thus favoring the spread of Hz-2V in the oviduct tissues of agonadal moths.

During the latter stages of virus replication in these tissues, the grossly enlarged nuclei fill with neatly packed virus particles, and infected cells often begin to lose their structural integrity. Some cells along the lumen appear to lyse and release either virus-filled "vesicles" or free virus particles into the lumen, the outcome possibly depending on whether or not the nuclear membrane remains intact. Infected cells that are not adjacent to the lumen may lyse and release virus particles into intercellular spaces, enabling the virus to then infect surrounding cells, resulting in clusters of infected cells. Infected nuclei were most often observed in patches of infected cells in the oviduct tissues of 8 and 10 dpp insects.

Examination of infected tissues in pharate adults suggests that the infection of oviduct tissues progresses from the cephalad region of the lateral oviducts to the common

oviduct, resulting in the cells of the common oviduct becoming infected days later than those in the lateral oviducts. In infected 6-7 dpp insects, infected cells were observed mainly in the cephalad region of the lateral oviducts, whereas no infected cells were observed in the common oviducts. Between 8 dpp and 10 dpp, there is a dramatic increase in the number of infected cells and an increase in the quantity of virus particles in the lumen of the cephalad region of the lateral oviducts. This exponential increase in the number of free virus particles in the lumina appears to result in the infection of more cells along the lumina of the lateral oviducts and eventually the common oviduct, and an increasing number of virus particles in the lumina that progress toward the caudal end of the malformed reproductive tract. As in the lateral oviducts, there is an increase in the number of cells with infected nuclei containing arrays of virus particles in the common oviduct tissues and an exponential increase in the number of free virus particles in the lumen of the common oviduct as the infected pharate adult matures.

By 10 dpp, as the pharate adult insect nears eclosion, the virus particles fill the lumina of the oviducts. The hypertrophied common oviduct is joined directly to the malformed cervix bursa of the bursa copulatrix, whereas the oviduct below the connection with the cervix bursa becomes constricted. Because of this abnormal morphology, large quantities of virus particles funnel through this larger opening into the malformed cervix bursa of the bursa copulatrix, causing the cervix bursa to swell with a mixture of concentrated virus and cellular debris. As the female pharate adult matures into an adult moth, this mass of virus separates into virus-filled spheres, which may subsequently be modified into the membrane-bound vesicles observed by Hamm *et al.* (1996) and Raina *et al.* (2000), before the spheres pass through the ductus bursa and

exude out of the vulva, ultimately coalescing into the viscous, viral plug over the reproductive opening described by Hamm *et al.* (1996).

The evolution of Hz-2V in *H. zea* has apparently culminated in the ability of the virus to reprogram the development and differentiation of the reproductive tissues in agonadal female moths. This reprogramming results in a proliferation of cells in these tissues that are propitious for virus replication, and an inhibition of the formation of the cuticular lining on the apical side of the cells. These developmental changes appear to enable Hz-2V to spread throughout the tissues and replicate to very high titers, ultimately forming a viral "waxy plug" over the opening of the vulva.

The role of this viral plug in the biology of Hz-2V is not known, but it is likely to be important in the horizontal and vertical transmission of the virus. Hz-2V infected female corn earworm moths produce two to three times more sex pheromone than uninfected female moths (Raina *et al.* 2000), possibly enhancing their ability to attract healthy male moths, which could become infected with Hz-2V from the viral plug while attempting to mate with the infected females.

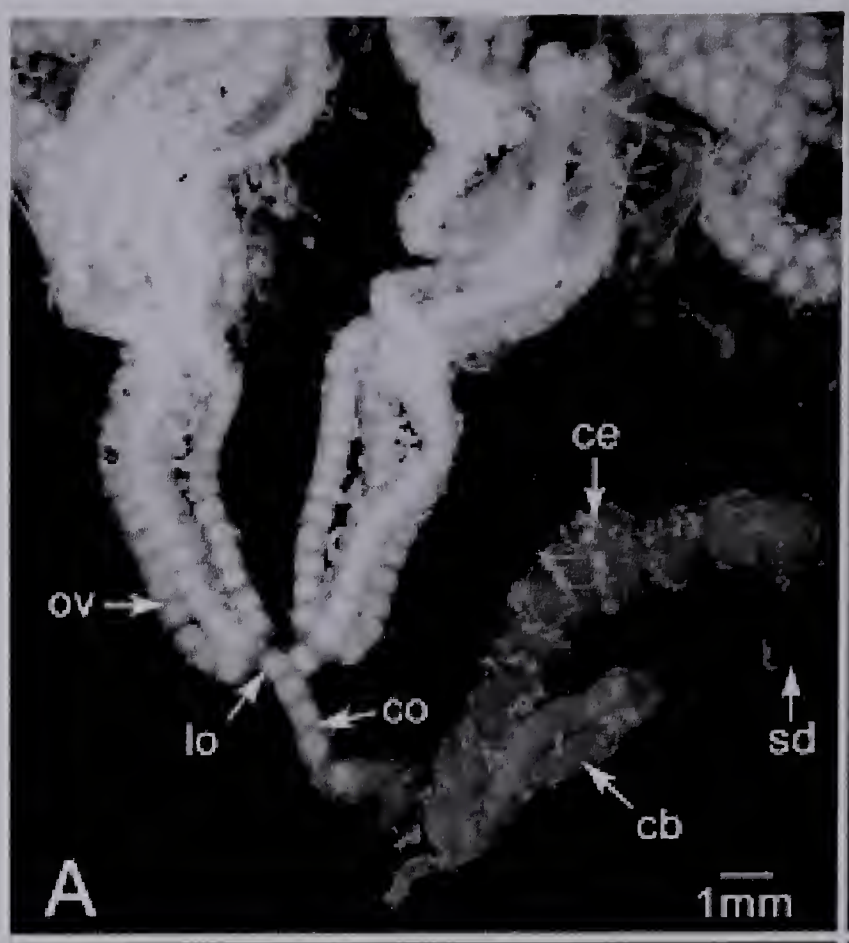
It is also possible that agonadal females may visit and contaminate substrates used by other life stages of *H. zea*. We observed both healthy and agonadal female moths pressing the end of their abdomens onto pitched substrates, perhaps for extra support, as they move about in observation chambers (data not shown). Other life stages of the insect could become contaminated or infected with the virus when they encounter or consume substrates contaminated in this manner.

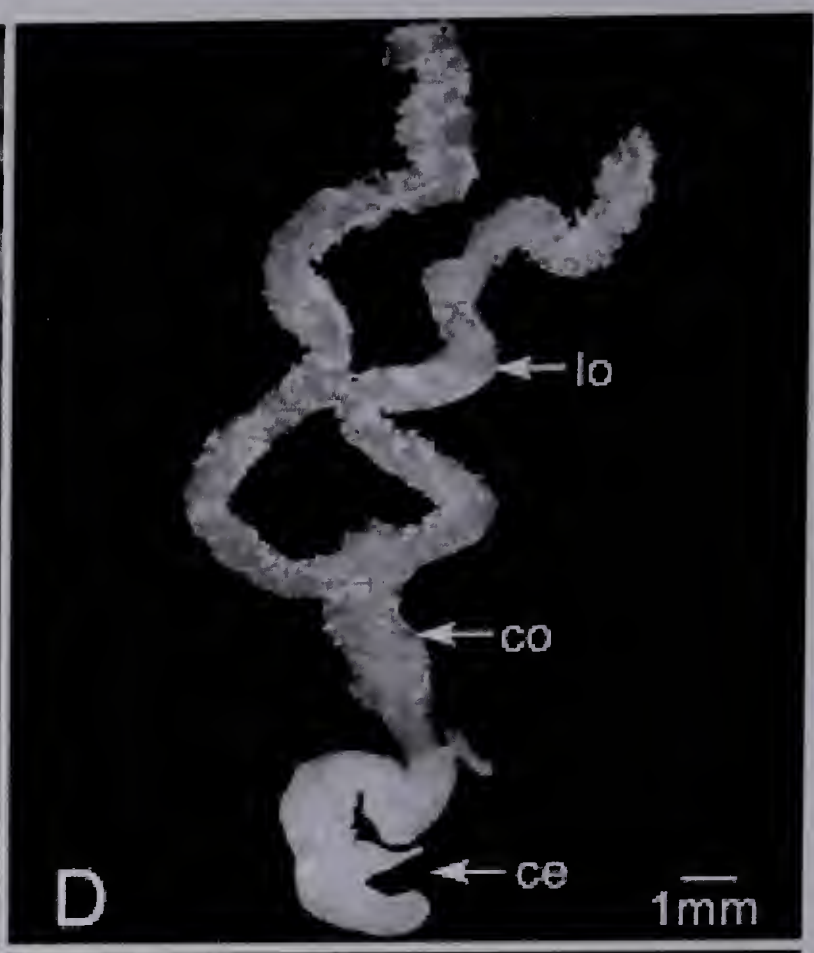
Table 3.1. The effect of the injection of Hz-2V into *H. zea* at different life stages.

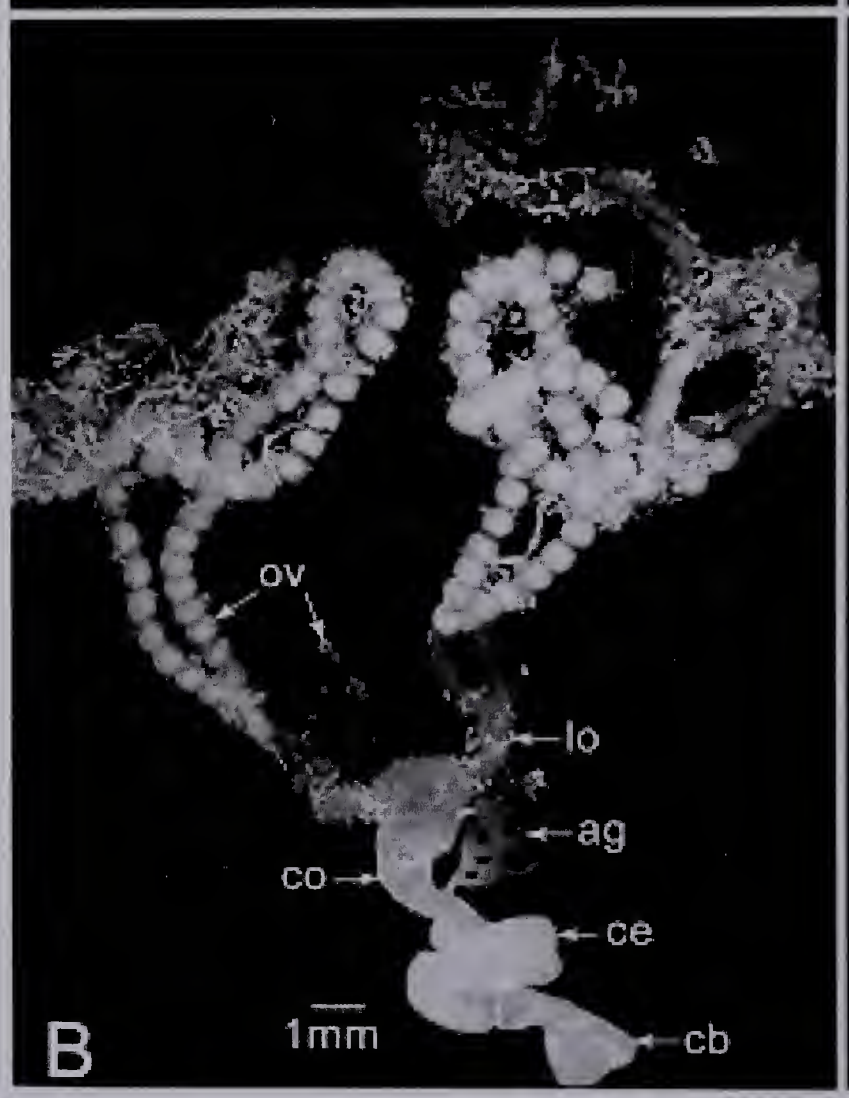
Life Stage	Reproductive Tissue				
	Com. Ovi.	Bursa cop.	Lat. Ovi.	Ovarioles	Eggs
4th instar	A	A	X	X	X
5th instar	A	A	A	X	X
6th instar	A	A	A	N/X*	N/X*
Prepupal	A	A	N	N	N
Pupal	N	N	N	N	N

N = normal development
A = abnormal development
X = tissues are absent or unrecognizable
* Some ovarioles missing or severely deformed.

Figure 3.1. Reproductive systems of adult female *H. zea* showing healthy, agonadal, and intermediate malformed tissues resulting from Hz-2V infection of different life stages. (A) Healthy female ovarioles (ov), lateral oviduct (lo), common oviduct (co), cervix bursa (ce), corpus bursa (cb), and seminal duct (sd). (B) Slightly malformed reproductive tissues of adult female infected with Hz-2V during the late 6th larval instar. (C) Malformed reproductive tissues of female infected during the late 5th larval instar. (D) Grossly malformed reproductive tissues of a typical agonadal female adult moth arising from an egg laid by an infected adult female. Note the difference in development of the lateral oviducts, common oviducts, cervix bursae, and corpus bursae in each of the panels.


ce
ov
lo
co
sd
cb
A
1mm


lo
co
ce
D
1mm


ov
lo
ag
co
ce
cb
B
1mm

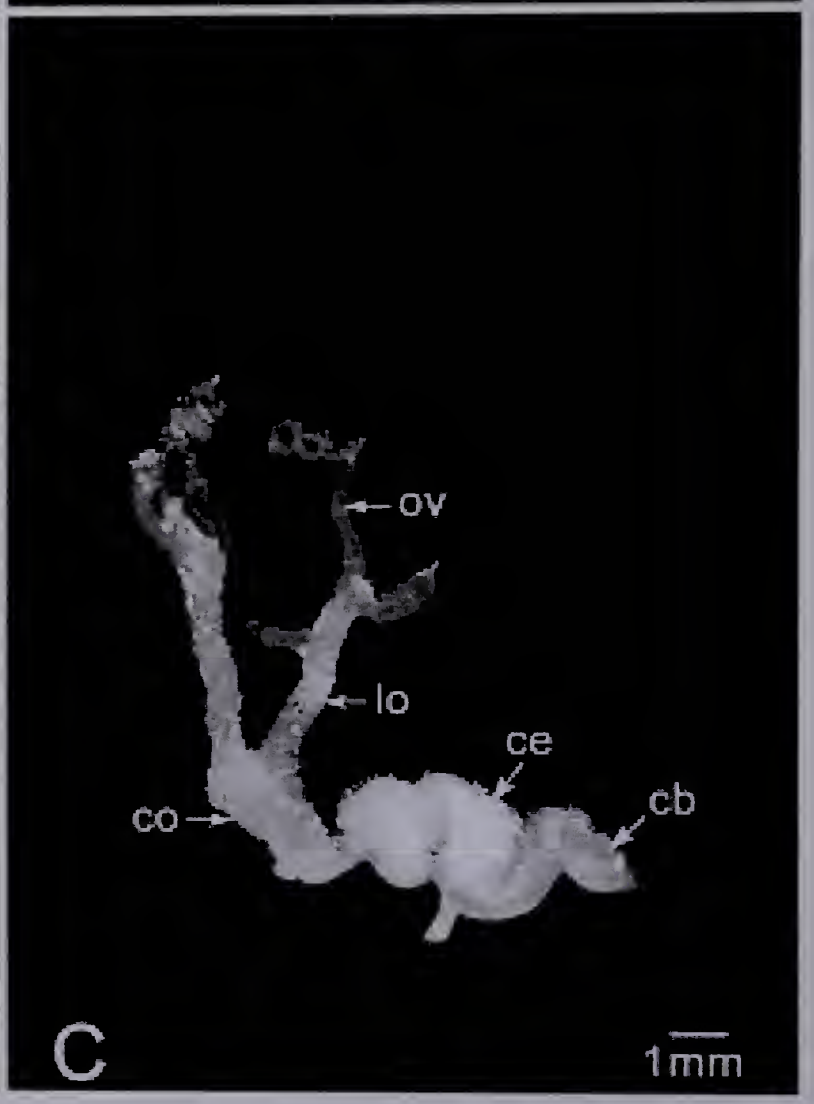

ov
lo
ce
co
cb
C
1mm

Figure 3.2. Healthy and agonadal reproductive systems of 4 dpp agonadal female *H. zea*. (A) Healthy ovarioles (ov), lateral oviduct (lo), common oviducts (co), accessory glands (ag), cervix bursa (ce), and corpus bursa (cb). (B) Grossly malformed and enlarged reproductive tissues of a 4 dpp agonadal female *H. zea* exhibiting a vast difference in the size of the oviduct tissues.

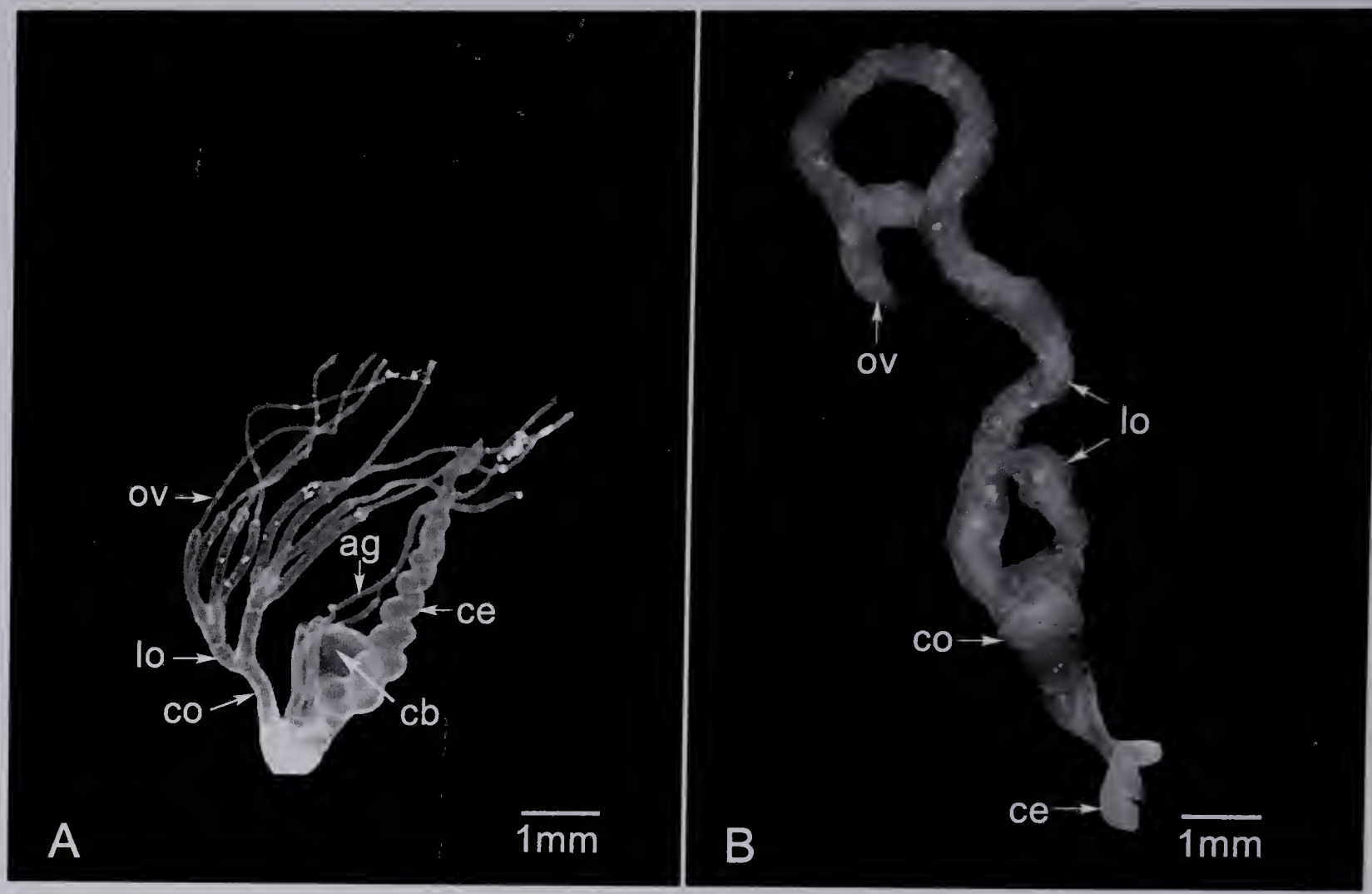

ov
ag
ce
lo
co
cb
A
1mm
ov
lo
co
ce
B
1mm

Figure 3.3. Electron micrographs showing the differences in development of oviduct tissues in pharate adult and adult female *H. zea*. (A) Healthy 7 dpp common oviduct showing a single layer of epithelial cells containing spherical to ovoid nuclei (N), and lined on the lumen (L) side by a well developed membranous layer (ML) comprised of a procuticle (P) and an epicuticle (E). Inset: Magnified section of the cuticular lining revealing more detail in the procuticle (P) and epicuticle (E). (B) Agonadal 4 dpp lateral oviduct malformed into tissues comprised of multiple layers of cells. Cells exposed to the lumen often formed microvilli (M), which projected into the lumen. Inset: Magnified section of the apical side of the cells revealing several microvilli projecting into the lumen. (C) Healthy adult lateral oviduct with mature membranous lining comprised of a thin procuticle lined by a thick epicuticle. (D) Cephalad end of healthy 10 dpp lateral oviduct showing diffuse membranous layer lining the lumen.

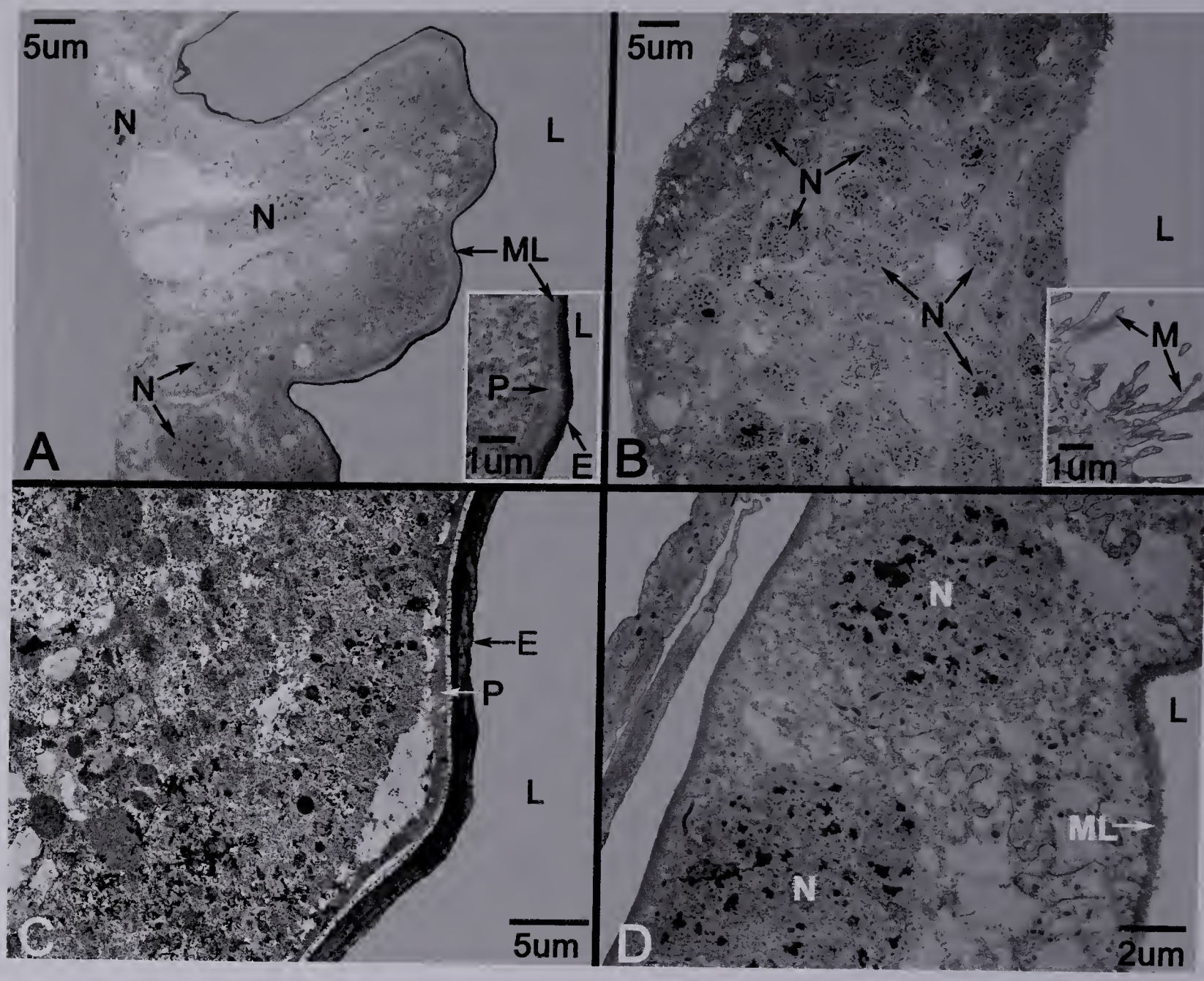

5um
N
N
L
ML
N
L
P
1um
E
A
5um
N
N
L
N
M
1um
B
E
P
L
5um
C
N
L
ML
N
2um
D

Figure 3.4. Electron micrographs of infected cells in oviduct tissues of agonadal female *H. zea*. (A) Lateral oviduct of an agonadal female 10 dpp showing numerous virus particles (V) filling the lumen (L) (inset, lower right) and organized in arrays in infected nuclei (Ni) (inset, upper right). Note the absence of a well-defined nuclear membrane surrounding the nucleus of the virus-infected cell on the right. (B) Common oviduct of a 10 dpp agonadal female showing cluster of cells containing infected nuclei (Ni) filled with virus particles. The infected cells are electron-dense and therefore stain more darkly than uninfected cells [cells with healthy nuclei (N)].

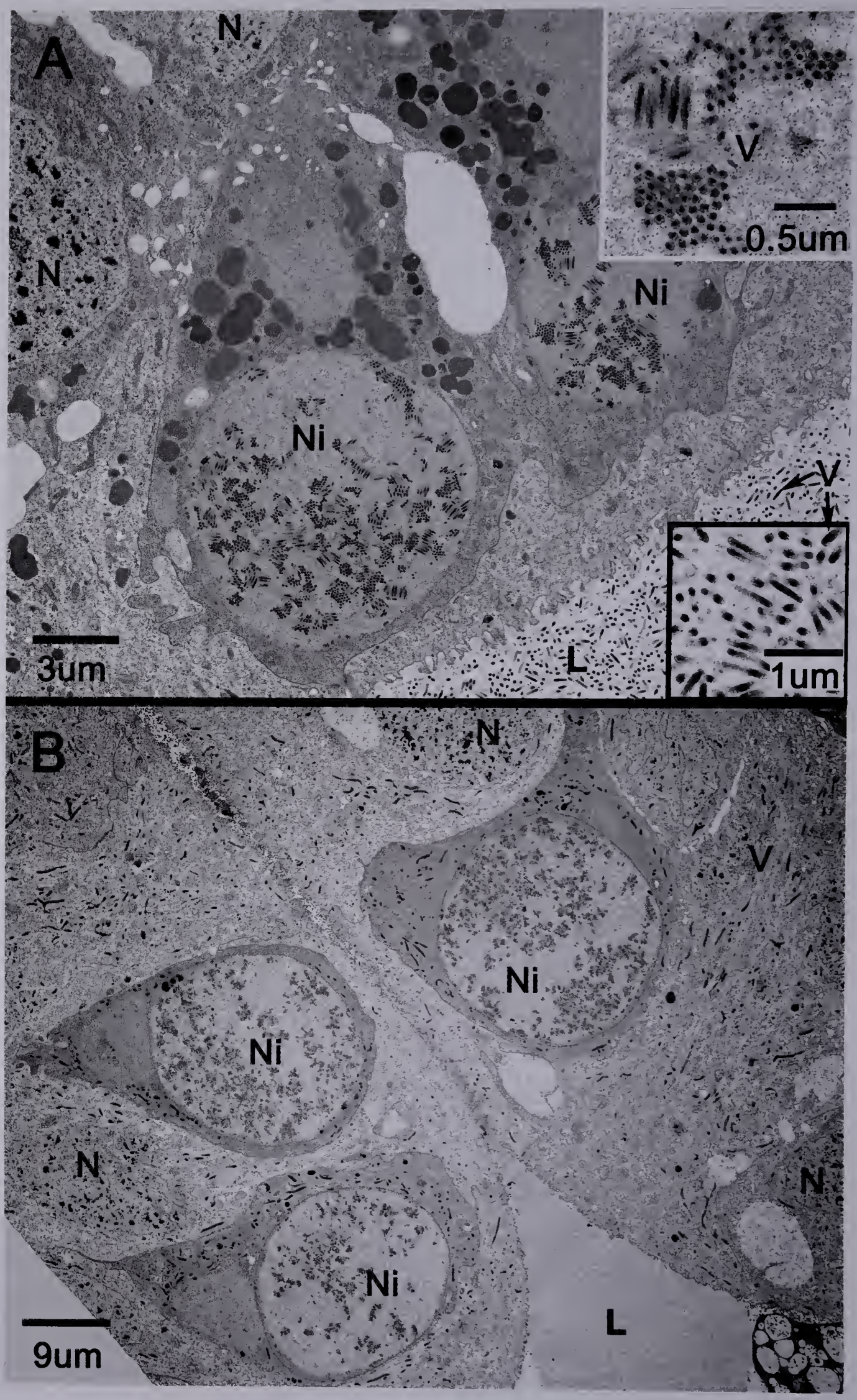

A
N
N
Ni
Ni
V
0.5um
V
3um
L
1um
B
N
V
Ni
Ni
N
N
Ni
L
9um

Figure 3.5. Electron micrographs of oviduct tissues showing an Hz-2V infected cell lysing, and vesicles filled with virus particles. (A) Lateral oviduct from an agonadal 10 dpp female showing an infected cell lysing and releasing virus particles (V) from the infected nucleus (Ni) into the lumen (L). Inset: Magnified section of the partially lysed, virus-filled cell, revealing tightly packed virus particles. (B) Lumen of the lateral oviduct from an agonadal 10 dpp female containing a large, virus-filled vesicle with a broken or degraded outer membrane, allowing virus particles to spill into the lumen. (C) Common oviduct from an agonadal 7.5 dpp female showing a virus-filled vesicle at a cellular junction along the lumen. This vesicle has also partially lysed, releasing virus particles into the lumen.

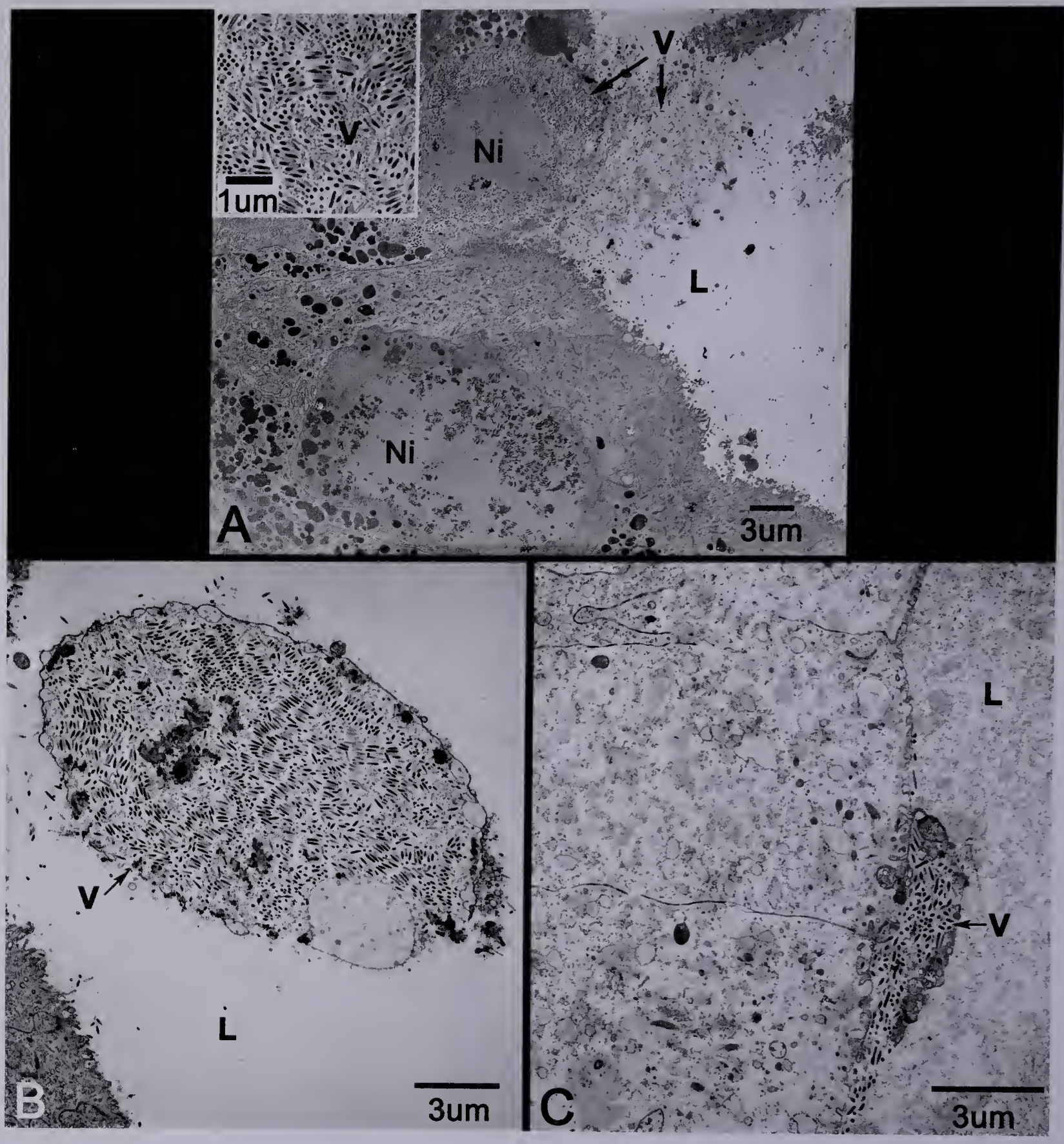

V
1um
Ni
V
L
Ni
A
3um
V
L
B
3um
L
V
C
3um

Figure 3.6. Electron micrographs of cervix bursae tissues from healthy and agonadal females. (A) Healthy adult cervix bursa showing a single layer of epithelial cells containing spherical to ovoid nuclei (N). Part of a thick procuticle (P) is also shown, appearing as alternating waves of light and dark tissue. The epicuticle is not shown. (B) The cuticular lining and the contents of the lumen of a cervix bursa from an agonadal 10 dpp female. The lining is composed of a comparatively thin procuticle and epicuticle (E), and an extensive mass of virus particles intermingled with a dark staining substance fills the lumen (L).

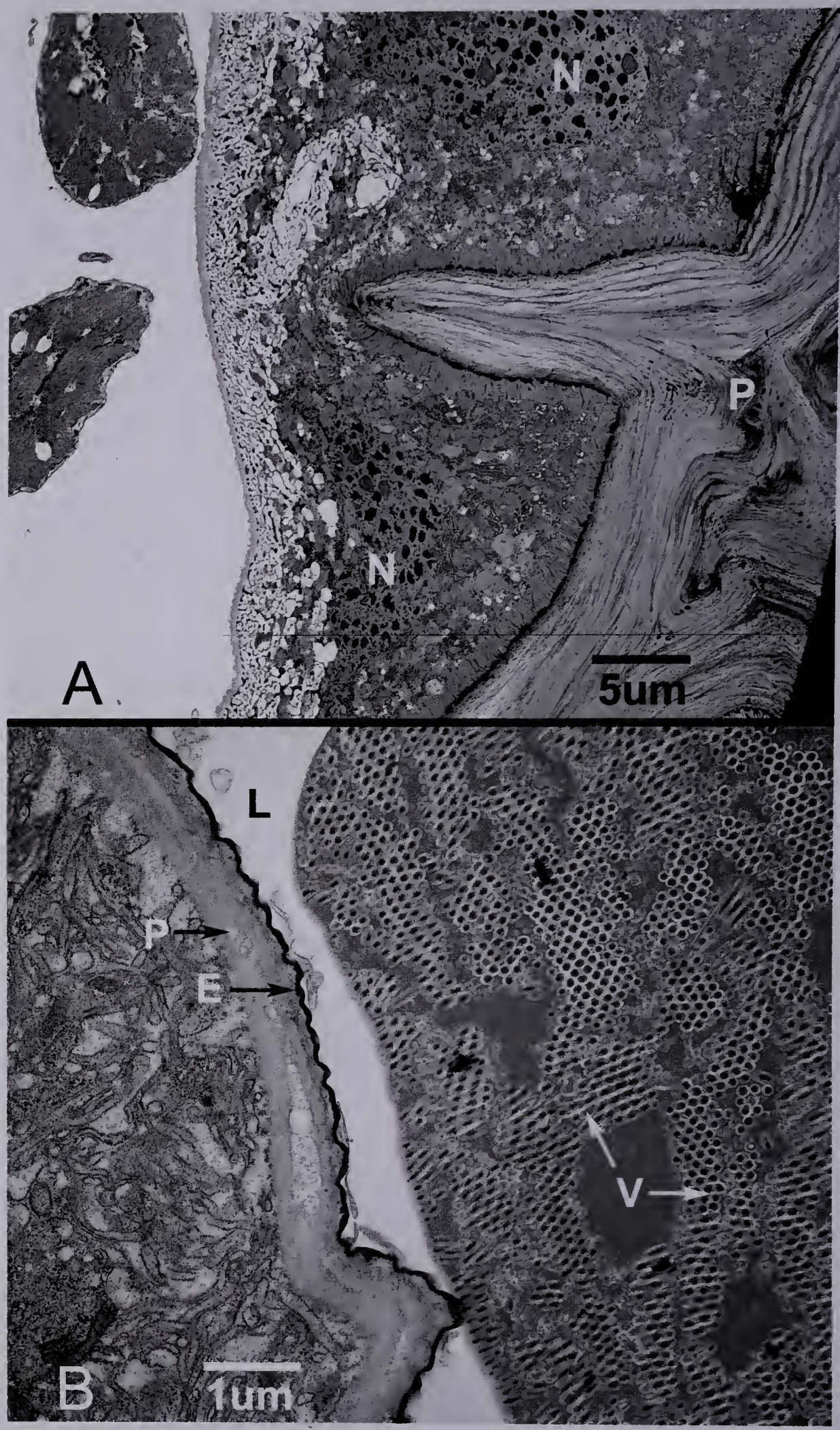
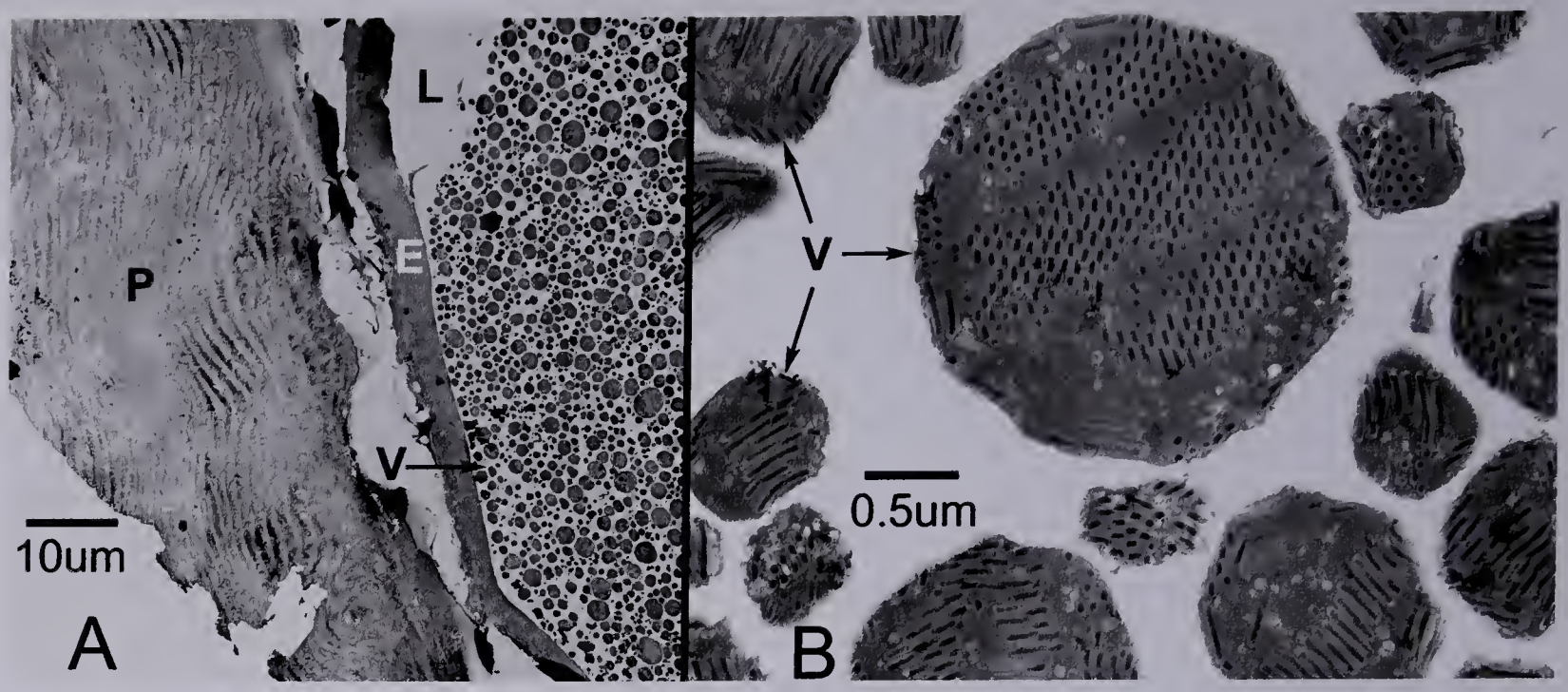

N
P
N
A
5um
L
P
E
V
B
1um

Figure 3.7. Electron micrographs of the cervix bursa from a newly emerged, agonadal adult female moth. (A) Part of the thick procuticle (P) and thinner epicuticle (E) are shown lining the lumen (L), which contains numerous spheres of various sizes comprised of virus particles (V) intermingled with a dark staining substance. (B) Higher magnification of the virus-filled spheres. Note the lack of a membrane surrounding the spheres.


L
E
P
V
10um
A
V
0.5um
B

CHAPTER 4

DOSE-RESPONSE

Introduction

Productive replication of Hz-2V in corn earworm moths, *Helicoverpa zea*, results in the abnormal development of the reproductive tissues of infected insects, and the proliferation of tissues that support the productive replication of the virus. These virus-infected adult moths are sterile and exhibit a condition that has been referred to as agonadal (chapters II and III). In spite of the sterility of these infected moths, the virus has been found to be maintained in a lab colony of corn earworms in the USDA-ARS in Stoneville, MS for several generations (Raina and Adams 1995; Hamm *et al.* 1996).

While examining progeny from eggs laid by infected female moths, Hamm *et al.* (1996) identified individuals that appeared healthy and were capable of transmitting Hz-2V to their progeny. Using PCR analysis, Lupiani *et al.* (1998) were able to detect viral DNA sequences in feral corn earworms from wild populations that appeared normal. These apparently healthy, infected moths were designated asymptomatic carriers of Hz-2V. The ability of Hz-2V to cause gross malformation of reproductive tissues and sterility in *H. zea*, yet be maintained in populations of moths reflects an important feature of the unique biology of this virus; that is the ability of the virus to establish persistent or latent infections in insects.

Hamm *et al.* (1996) presented evidence from experimental matings involving asymptomatic female moths and healthy males that showed the proportion of agonadal progeny arising from eggs laid on successive oviposition days increased rapidly with each oviposition day, suggesting a change in viral activity in the asymptomatic female.

They proposed that the outcome of virus infection in the progeny was related to virus dose, such that eggs laid on early oviposition days received a low virus dose resulting in more asymptomatic virus carrier moths, whereas those arising from later oviposition days received a high virus dose and developed into agonadal moths. These findings suggest that Hz-2V is able to exist in a persistent or latent state in some corn earworms and become induced into productive replication at a specific time in the development of the insect. During their experiments, Hamm *et al.* (1996) were unable to accurately determine and control the virus dose female moths received and they were unable to directly detect females that were asymptomatic carriers of the virus.

Raina *et al.* (2000) showed that it was possible to inject Hz-2V into healthy female corn earworm moths, and upon mating with healthy male moths, produce asymptomatic carrier and agonadal progeny. They found that about half of all of the progeny produced by females that were infected with a moderate virus dose exhibited the agonadal condition and that about 90% of the remaining apparently normal progeny actually carried viral DNA sequences detectable by PCR. This Hz-2V injection data suggests that this approach could be used to experimentally produce females that mimic the asymptomatic carrier females described by Hamm *et al.* (1996).

Studying asymptomatic carrier females and their progeny should convey a better understanding of Hz-2V infection in asymptomatic carrier moths, including virus transmission to progeny and the effects of virus dose on the outcome of the infection. It is therefore important to be as precise as possible in measuring virus infectious dose. For these reasons, the effect of injected virus dose on the outcome of the infection in the corn earworm moth was carefully examined using a cell culture system that allows for the

precise measurement and control of infectious virus dose, and the PCR approach developed by Lupiani *et al.* (1998) for detecting the presence of asymptomatic carriers of Hz-2V.

Previously reported data on the replication of Hz-2V *in vitro* (Lu and Burand 2001) and on the replication *in vivo* in the reproductive tissues of agonadal female moths (chapter III) strongly indicate a 24 hour virus replication cycle occurs in *H. zea*. These data indicate that virus levels in the infected female moth increase exponentially on a daily basis. Thus, in order to more accurately determine the effects of virus levels on the outcome of the infection in progeny, data were collected and analyzed for each oviposition day for the progeny of females infected at precise virus doses.

The results of this study represent strong evidence that the initial dose of Hz-2V used to infect female moths and the level of replication of the virus in these moths reproductive tissues determine whether progeny insects develop into agonadal moths or asymptomatic carriers of Hz-2V, likely by affecting the level of virus transmitted to the progeny eggs prior to oviposition.

Materials and Methods

Source of Insects and Virus

Corn earworm larvae used to start a laboratory colony of healthy *H. zea* were obtained from an USDA-ARS in Stoneville, MI were. Insects were reared on artificial diet and maintained as outlined in chapter II.

Hz-2V for infecting female moths was prepared as described previously and purified via sucrose gradient centrifugation (Burand and Lu 1997).

Injection of Adults

Newly emerged adult female moths were prepared and injected with Hz-2V as outlined previously in chapter II. The female moths were divided into four dose groups, each of which were infected with Hz-2V at one of the following concentrations of 2 x 10^5, 2 x 10^6, 2 x 10^7, and 2 x 10^8 $TCID_{50}$ units.

$TCID_{50}$

Tn368 cells were cultured as per Burand and Lu (1997) and 100 µl of cell culture medium containing 8 x 10^4 Tn368 cells were seeded into each well of a 96-well plate. Between 6 and 13 serial dilutions were made from each virus sample assayed and 10 or 20 wells were plated with 10 µl for each dilution. Plates were incubated at 27°C for 3 to 4 days and examined for the appearance of cytopathic effect (CPE). The numbers of wells with CPE were counted and the $TCID_{50}$ calculated (King and Possee 1992).

DNA Extraction

DNA was extracted from the reproductive tissues of adult moths by first homogenizing dissected tissues in 200 µl of TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0) followed by a 2-minute incubation in a boiling water bath. The homogenate was chilled on ice, after which Ribonuclease A (10 µg/µl) was added to each sample, which was then incubated at room temperature for 15 min. Next the samples were clarified by centrifugation at 15,600 x g for 2 min., and 2 µl of each was used for PCR analysis.

Viral DNA used as template for PCR reactions was extracted from purified virus using 1% SDS in TE containing 1 mg/ml Protease K as outlined by Burand and Lu (1997).

PCR Amplification of Viral DNA Sequences

Two sets of primers were used to amplify Hz-2V genomic DNA to prepare a probe for use in slot-blot analysis of insect reproductive tissues. The first set (P4-1, 5'-GCACGATTCGTAATGTTC-3'; and P4-2, 5'-GCACACCTATCAATCACC-3') was designed to amplify a 434 bp sequence of the Hz-2V genome (Lupiani *et al.* 1998). PCR reactions using P4-1 and P4-2 primers were brought to a final volume of 20 µl using the Bioneer AccuPower® PCR reagent premix kit with 1 unit of Taq DNA polymerase. Reactions were carried out in 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, and 40 mM KCl, containing 250 µM of each of the 4 dNTP's, with 100 pM of P4-1 forward and P4-2 reverse primers and 10 ng of purified viral DNA as template. The primer set and reaction

conditions were also used to amplify viral DNA sequences in approximately 100 ng of DNA from reproductive tissues of moths thought to be asymptomatic carriers of Hz-2V.

The second set of primers (P4-3, 5'-GCTGTGCTGTACAAGTGC-3'; and P4-4, 5'-CCCTTGACGATCCCTTTTG-3') was designed to amplify a 350 bp region directly interior to that of the P4-1 and P4-2 amplified sequence. These primers were used to generate a digoxigenin (DIG)-labeled probe for Hz-2V to be used in slot blot hybridization assays. PCR reactions for production of the DIG-labeled probe were carried out in a final volume of 50 µl using the Boehringer Manheim DIG High Prime DNA Labeling and Detection Kit, with 1X concentrations of Taq Polymerase buffer (100 mM Tris-HCl pH 8.0, 500 mM KCL pH 8.3, and 25 mM $MgCL_2$), 100 pM of both P4-3 and P4-4 primers, a hexanucleotide mixture containing DIG-labeled dUTP (2 mM dATP, dCTP, dGTP, 1.3 mM dTTP, and 0.7 mM alkali labile DIG-11 dUTP pH 7.0), and 100 pM of Hz-2V genomic DNA. The DIG-labeled PCR product was purified on a 0.8% agarose gel using the Qiagen gel electrophoresis purification kit.

Both PCR reactions for amplification of the virus DNA in tissue samples and for the production of the viral DNA probe consisted of 30 cycles of a DNA denaturation step at 95 °C for 1 min., a primer annealing step for 1 min. at 55 °C, and a 1 min. primer extension step at 72°C.

Detection of a Viral DNA Sequence by Slot-blotting

To prepare the DNA for slot-blot analysis, 15 µl of the P4-1 and P4-2 PCR-amplified DNA from insect samples was denatured by incubating with NaOH (0.4 M)/ EDTA (10 mM, pH 8.2) at 100°C for 10 min., then applied to a Hybon-N+ membrane

prewashed with 500 µl 5X SSC buffer (0.6 M NaCl, 60 mM Na citrate pH 7.0) in a Manifold II slot-blotter (Schleicher & Schuell). After applying the DNA, the membrane was baked at 88°C for 2 hrs. under vacuum pressure, and prehybridized for 6 hrs. at 42°C in 50% formamide prehybridization buffer (5X SSC, 0.1% (w/v) N-laurylsarcosine, 1% (w/v) Na_2-dodecylsulfate, 2% Blocking reagent (Boehringer-Manheim), and 50% formamide. Slot blots were hybridized with 150 ng DIG-labeled Hz-2V probe at 37°C for 12-14 hrs. Following washing, chemiluminescent detection was carried out as recommended by the DIG High Prime Labeling and Detection Kit Manual for DNA Hybridization (Boehringer Mannheim). The Boehringer Mannheim protocol, performed using x-ray film, produced dark blots corresponding to viral DNA from sample wells on the slot-blot membrane.

Analysis of PCR Products by Agarose Gel Electrophoresis

In order to confirm that the PCR products that hybridized to the viral DNA probe contained an amplified DNA fragment of the appropriate size (434 bp), representative samples were analyzed by electrophoresis on 0.8% agarose gels with 0.5X TBE buffer at 100 volts for approximately 1 hr., then stained with Ethidium Bromide to visualize DNA bands under ultraviolet light.

Results

More than 1600 progeny moths resulting from eggs laid by 4 groups of females infected with 2×10^5, 2×10^6, 2×10^7, or 2×10^8 $TCID_{50}$ units of Hz-2V, were dissected and the reproductive tissues examined for signs of virus pathology. Moths that had reproductive tissues that appeared to be normal but tested positive for Hz-2V DNA by PCR analyses (Figs. 1 and 2) were considered asymptomatic carriers of the virus. At all virus doses tested the numbers of agonadal moths, asymptomatic carriers, infected individuals (the sum of agonadal and asymptomatic carriers), and uninfected progeny moths hatching from eggs laid on each oviposition day were recorded.

The analysis of these results showed that the percentage of total infected progeny (asymptomatic carriers and agonadal moths) at all doses tested increased with each successive oviposition day, and the level of infected moths increased as virus dose increased from 2×10^5 to 2×10^8 $TCID_{50}$ units (Fig. 3). For progeny hatching from eggs on oviposition day one, the highest percentage of infected progeny (85%) was produced by females infected with the highest virus dose (2×10^8), whereas the lowest percentage (59%) was produced by females infected with the lowest dose of virus (2×10^5 $TCID_{50}$).

Virus-infected progeny were further separated into the percentages of agonadal and asymptomatic carrier progeny moths arising from eggs laid on each oviposition day by females infected with different virus doses (Fig. 4). No agonadal insects arose from eggs laid on oviposition day one by females infected at the two lowest virus doses, while about 14% of the progeny from eggs laid at this time by females infected at the highest dose were agonadal. Approximately 71% to 74% of F1's from insects infected with the

highest doses (2×10^8 and 2×10^7) and 91% to 95% of F1's from females infected with the lower doses (2×10^6 and 2×10^5) were asymptomatic carriers.

For all F1 insects hatching from eggs laid on day two, the percentage of agonadal moths increased with increasing virus dose and the percentage of asymptomatic carriers at each dose declined. Again, the highest number of agonadal progeny (approximately 72%) hatching from eggs laid on day two came from females that received the highest virus dose, whereas the level of day two agonadal females was just over 7% at the lowest dose. At all doses, 94% to 99% of the eggs laid on day three and 100% of eggs laid on day four gave rise to agonadal moths.

In order to examine the relationship between the two types of infections and to emphasize the effects of virus dose upon the proportions of asymptomatic and agonadal infections, percentages of asymptomatic carriers and agonadal progeny were calculated from total infected progeny for only the highest and lowest dose (Fig. 5). The trend in the two types of infected progeny insects follows the same general pattern for both virus doses relative to oviposition day. That is, at both doses the percentage of agonadal progeny increases with oviposition day as the percentage of infected insects that are asymptomatic carriers of Hz-2V decreases. At the highest dose, the proportion of agonadal insects starts out higher (~12%) on the first oviposition day than that of agonadal progeny of females infected at the lowest dose (0%), and rises more quickly to almost 72% of the progeny from eggs laid on oviposition day two, compared to only about 7% of the progeny arising from day two eggs laid by females infected at the lowest dose. These data clearly show that there is a direct correlation between virus dose and the

relative percentage of agonadal and asymptomatic progeny. That is, increasing the virus dose causes an increase in the percentage of agonadal progeny, but a decrease in the percentage of asymptomatic progeny.

Discussion

Injecting Hz-2V into female moths results in experimentally infected insects that resemble asymptomatic females and females that have become infected during copulation, such as the females infected during mass-matings with infected males during transmission experiments conducted by Hamm *et al.* (1996). These infected moths appear normal, are fertile, and can transmit the virus to progeny that result from mating. Some of the progeny moths that result from these matings do not carry any detectable viral DNA sequences, others are sterile with malformed reproductive tissues, and others are asymptomatic carriers of the virus. This variety of infections suggests that the virus is not initially present in all of the eggs of infected females, but is transmitted transovarially to some of the eggs sometime prior to oviposition. This idea is important in that it suggests that the dose or titer of virus transmitted from the female parent moth to the developing oocytes is not constant, and that the virus dose that each oocyte receives determines the outcome of the infection when these progeny insects mature into adult moths.

In the results presented in Figure 4, the percentage of agonadal progeny resulting from eggs laid on oviposition day 1 by female moths infected with Hz-2V increased as the dose of Hz-2V used to infect the female moths was increased. Progeny arising from eggs laid on oviposition day 2 also exhibited this correlation between virus dose and percent agonadal progeny. This indicates that the titer of the virus present in the experimentally infected female moths determines the amount of virus that is transmitted to their eggs, and this titer of Hz-2V is directly correlated to the percentage of agonadal progeny arising from eggs laid by these infected females. If the dose of Hz-2V used to infect a female moth is increased, a corresponding increase is observed in the proportion

of agonadal progeny arising from eggs laid by that moth on each oviposition day, and subsequently, in total agonadal progeny arising from all eggs laid by that female.

The percentage of agonadal progeny also increases with each successive oviposition day to between 94% and 99% agonadal progeny by day 3 at the four virus doses tested, and all progeny moths arising from eggs laid on oviposition day four in all groups were agonadal. Based on the correlation between virus titer and percent agonadal progeny discussed in the previous paragraph, the increase in agonadal progeny per oviposition day is likely due to an increase in the titer of virus transmitted to the eggs, suggesting that the titer of virus increases in the parent female moths with each successive oviposition day.

Studies of Hz-2V replication *in vitro* revealed a rapid increase in virus titer by 24 hours post infection in Tn-368 and Ld-652Y cells (Burand and Lu 1997; Lu and Burand 2001). Hz-2V replication *in vivo* in the epithelial cells of agonadal female oviduct tissue has been described previously in the results section of chapter III. The level of virus detected in these tissues increased dramatically between 8 dpp, measured from the day the last larval exuviae was shed, and 10 dpp. It is likely that the large increase in virus over a 24 hour cycle observed *in vitro* also occurs *in vivo*, resulting in a daily exponential increase in the titer of Hz-2V in the oviduct tissues of experimentally infected female moths. The increase in virus in these tissues probably results in increased titers of virus being transmitted to the progeny with each successive oviposition day and ultimately in the patterns of infection reported here.

Based on this replication data, if low virus doses result in asymptomatic carrier moths, and high virus doses produce agonadal moths, then asymptomatic carriers would

likely arise from eggs produced on the earliest oviposition days and decrease with each day as the virus titer in the parent female moth increases. In fact, the percentage of asymptomatic carrier progeny in this experiment does decrease with each successive oviposition day to 0% by day 4. The percent asymptomatic carriers is highest in progeny that most likely receive the lowest virus dose, specifically progeny from oviposition day 1 and progeny arising from the parent female moths that were experimentally infected with the lowest doses of virus. This is the opposite of what was observed for agonadal progeny, which is at its highest level at the highest virus dose, specifically on the later oviposition days (days 4 or greater) and in progeny arising from parent female moths that were infected with the highest virus dose. Interestingly, the lowest percentage of asymptomatic carrier progeny arose from eggs laid by the groups of female moths that received the highest virus doses of Hz-2V (Fig. 4).These data suggest that the virus dose transmitted by infected female moths to their developing eggs determines whether the progeny develop the agonadal condition or become asymptomatic carriers of Hz-2V.

The results clearly show that there is a correlation between virus dose and the relative proportions of agonadal and asymptomatic carrier progeny. Increasing the virus dose used to infect parent female moths causes an increase in agonadal progeny and total infected progeny, and a corresponding decrease in asymptomatic carrier progeny. The specific processes that regulate these developmental changes are currently not known. Data presented in chapters II and III showed that injecting virus into larvae by the late 4th instar produced moths whose reproductive tissues resembled those of agonadal moths, whereas insects injected later in development resulted in progressively less malformations. The data presented here together with the life stage injection data

presented in chapters II and III suggests that a minimum titer of Hz-2V is needed at a key point in larval development to produce a viral factor(s) within the larval tissues at a threshold level required to reprogram the development and differentiation of the reproductive tissues into the agonadal structure. If this threshold is equaled or exceeded at this point in development, the progeny would exhibit the agonadal condition. However, if this threshold level is not attained, then the reproductive tissues likely would not be reprogrammed and the infected insect would become an apparently normal, fertile, asymptomatic carrier of Hz-2V.

The evolution of Hz-2V infection in *H. zea* has resulted in the ability of the virus to produce two different types of infections in the insects that enable the virus to replicate to high titers in the reprogrammed reproductive tissues in sterile agonadal moths, while maintaining itself in a population at very low titers in fertile asymptomatic carrier moths. Hz-2V is present in a persistent state in these asymptomatic carriers, and does not appear to enter productive replication until the late pupal or early adult life stage, possibly upon mating. If all Hz-2V infected moths developed the sterile, agonadal condition, this might lead to the extinction of the infected local population of moths, as well as the virus. The production of asymptomatic carrier moths ensures that some fertile, infected moths exist that can mate and produce infected progeny, enabling an Hz-2V-infected population to sustain itself, as in the case of the Stoneville colony.

The results presented in this chapter suggest that eggs laid on the initial oviposition days by asymptomatic carrier females receive either no or very low titers of virus, insufficient to produce the sterile, agonadal condition and instead resulting in fertile, uninfected or asymptomatic carrier moths. Eggs laid by infected females on the

latter oviposition days receive higher doses of virus due to productive replication in the reproductive tissues of the female asymptomatic carrier moths, resulting in progeny with the agonadal condition, which is most favorable for producing high titers of Hz-2V. These high virus titers in agonadal female moths lead to the formation of viral "waxy" plugs, which likely play a key role in horizontal transmission of the virus to healthy insects in the population during mating attempts as discussed in chapter III.

By controlling the infectious dose of Hz-2V administered to a female moth and by selecting progeny from specific oviposition days, it is possible to predict and control the percentages of progeny agonadal and asymptomatic carrier moths resulting from eggs laid by that female. This has implications for research as well as for using this virus for biological control. These results could be used to design an experiment to produce predominantly agonadal adults for the purpose of propagating Hz-2V *in vivo*, or produce predominantly asymptomatic carriers of Hz-2V for use in research on the persistence of the virus and the induction of productive virus replication in infected moths.

Figure 4.1. Slot-blot hybridization results of DNA extracted from reproductive tissues of corn earworm moths. DNA was extracted, amplified via PCR, transferred onto a nylon membrane, and allowed to hybridize with the DIG-labeled viral DNA probe. Dark blots are indicative of asymptomatic carrier moths (As), whereas blots that were blank or very light were from insects considered to be healthy, uninfected moths (N).

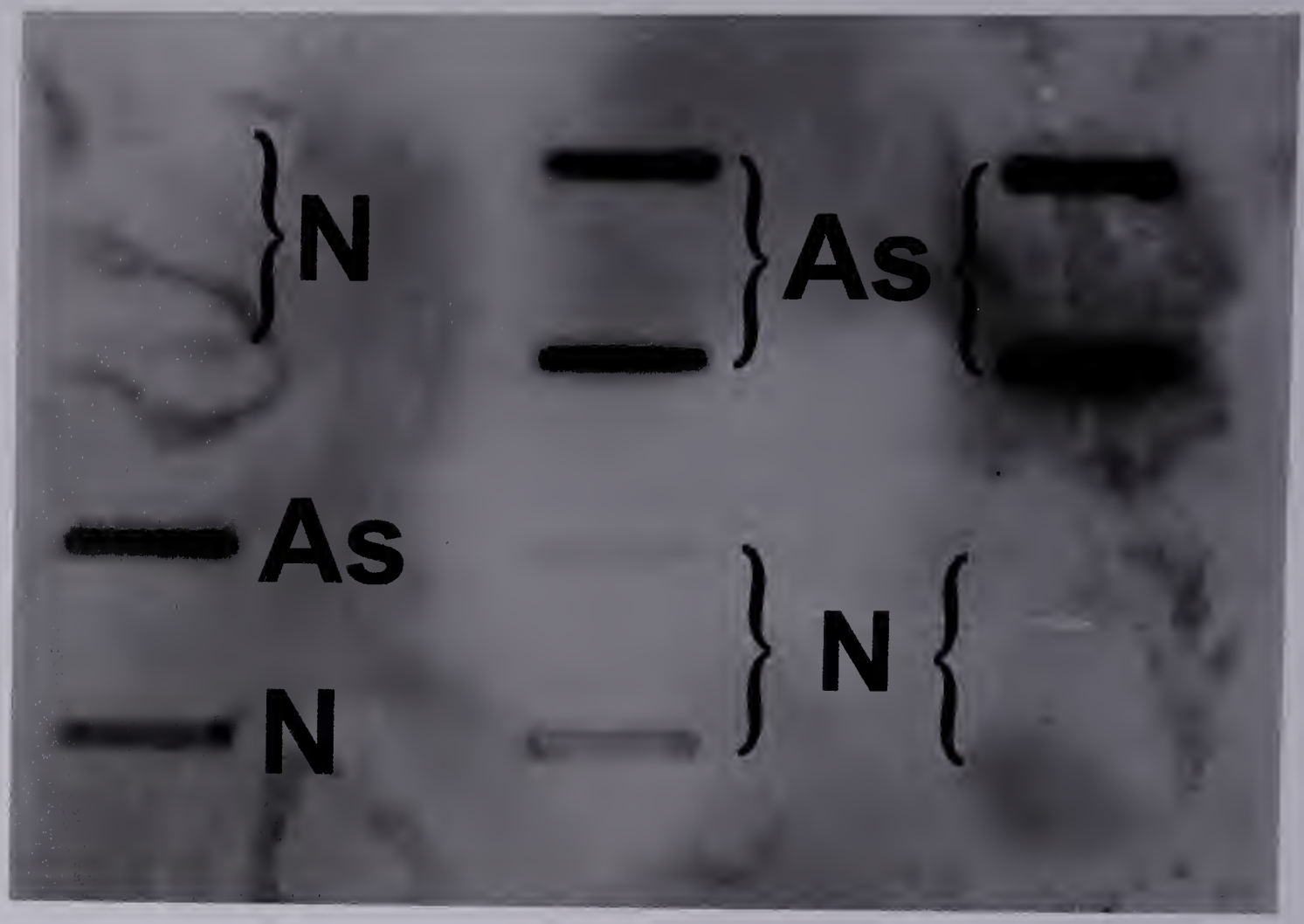

N
As
As
N
N

Figure 4.2. EtBr-stained agarose gel of PCR results from DNA extracted from the reproductive tissues of corn earworm moths via alkaline lysis with Proteinase K. The first lane contained purified viral DNA (PV). Lanes 2 and 3 contained PCR product obtained from healthy, uninfected colony moths (N). DNA samples extracted from progeny corn earworm moths infected with Hz-2V were placed in lanes 4-8.

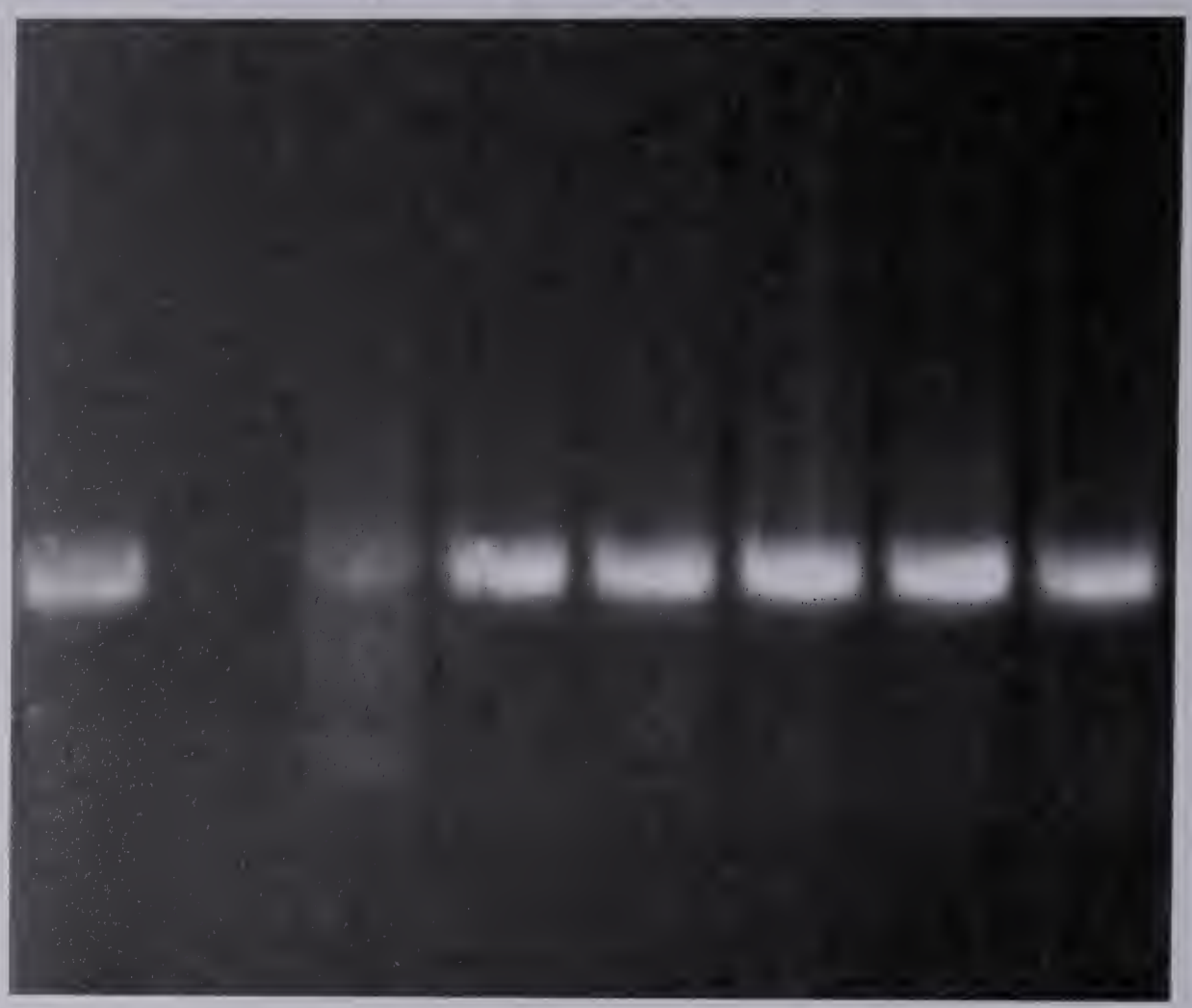

PV
N
AG
1
2
3
4
5
6
7
8

Figure 4.3. Results from all infected progeny arising from eggs laid on the first 4 oviposition days by parent female moths experimentally infected with 2×10^5, 2×10^6, 2×10^7, and 2×10^8 $TCID_{50}$ units of Hz-2V. Infected progeny include agonadal and asymptomatic carriers of Hz-2V.

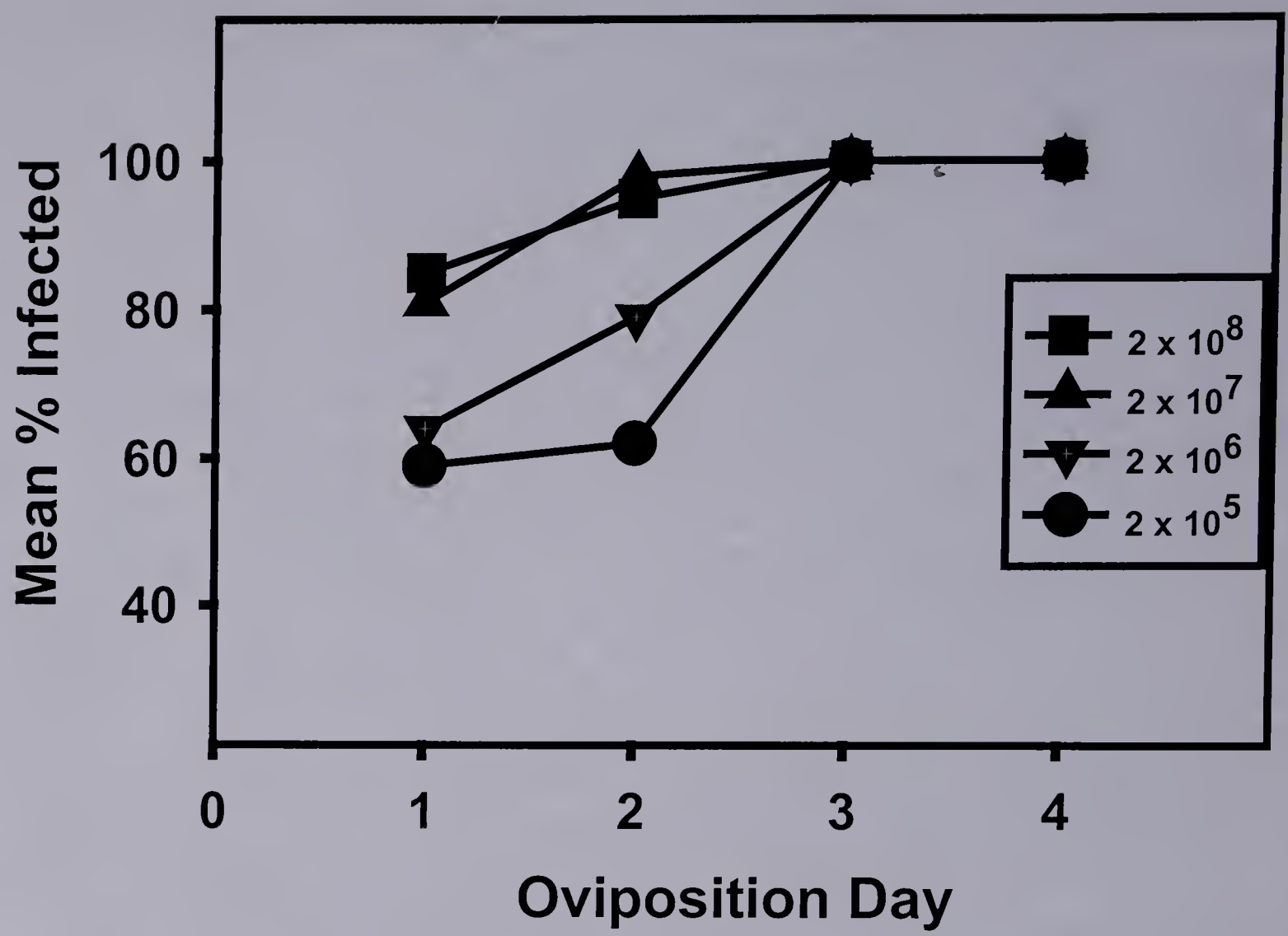

Mean % Infected
100
80
60
40
0
1
2
3
4
Oviposition Day
2 x 10^8
2 x 10^7
2 x 10^6
2 x 10^5

Figure 4.4. Agonadal (AG), asymptomatic carrier (AS), and uninfected progeny of parent female moths experimentally infected with 2×10^5, 2×10^6, 2×10^7, and 2×10^8 $TCID_{50}$ units of Hz-2V. Asymptomatic carrier progeny are represented by broken lines in the figure, and agonadal progeny by solid lines.

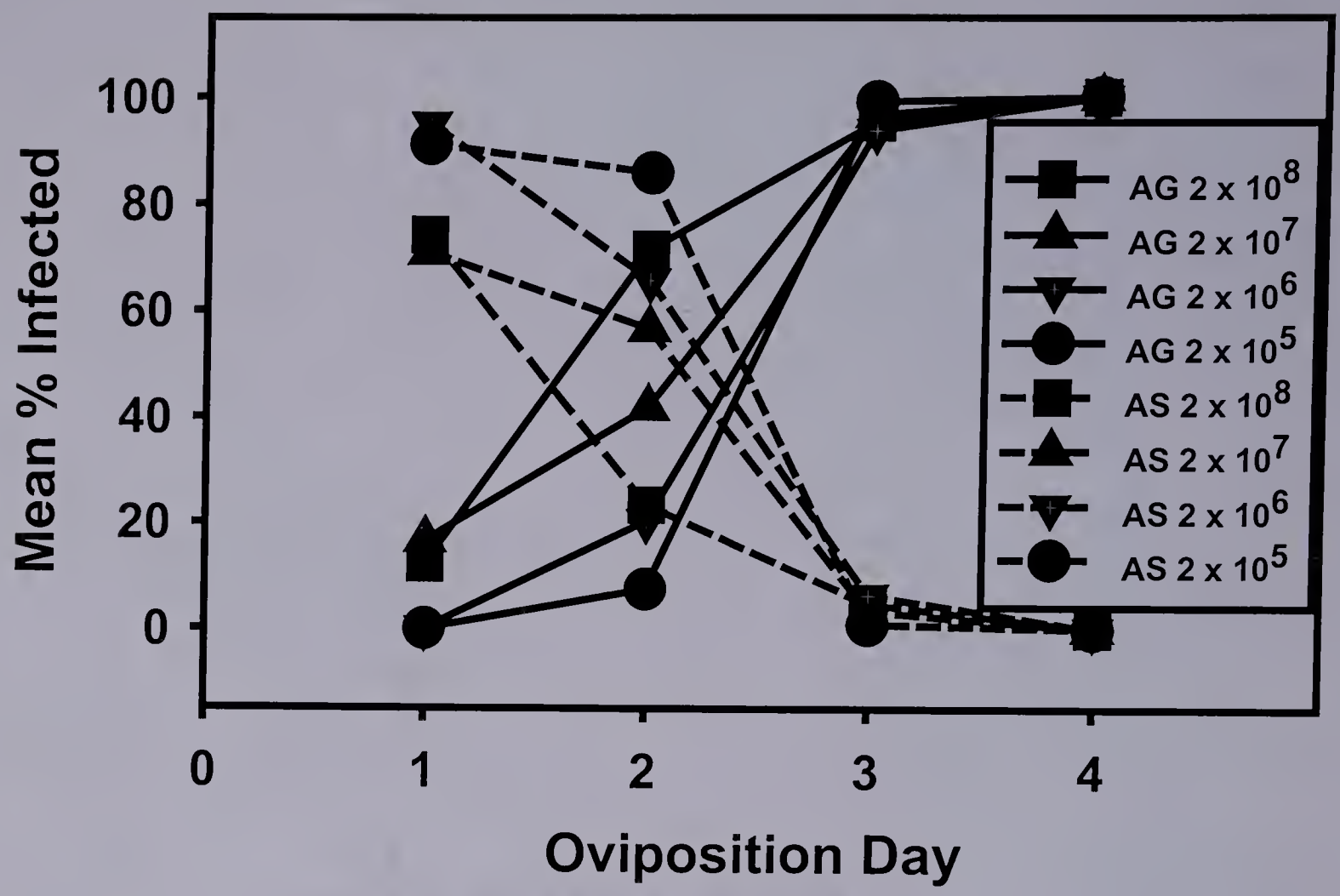
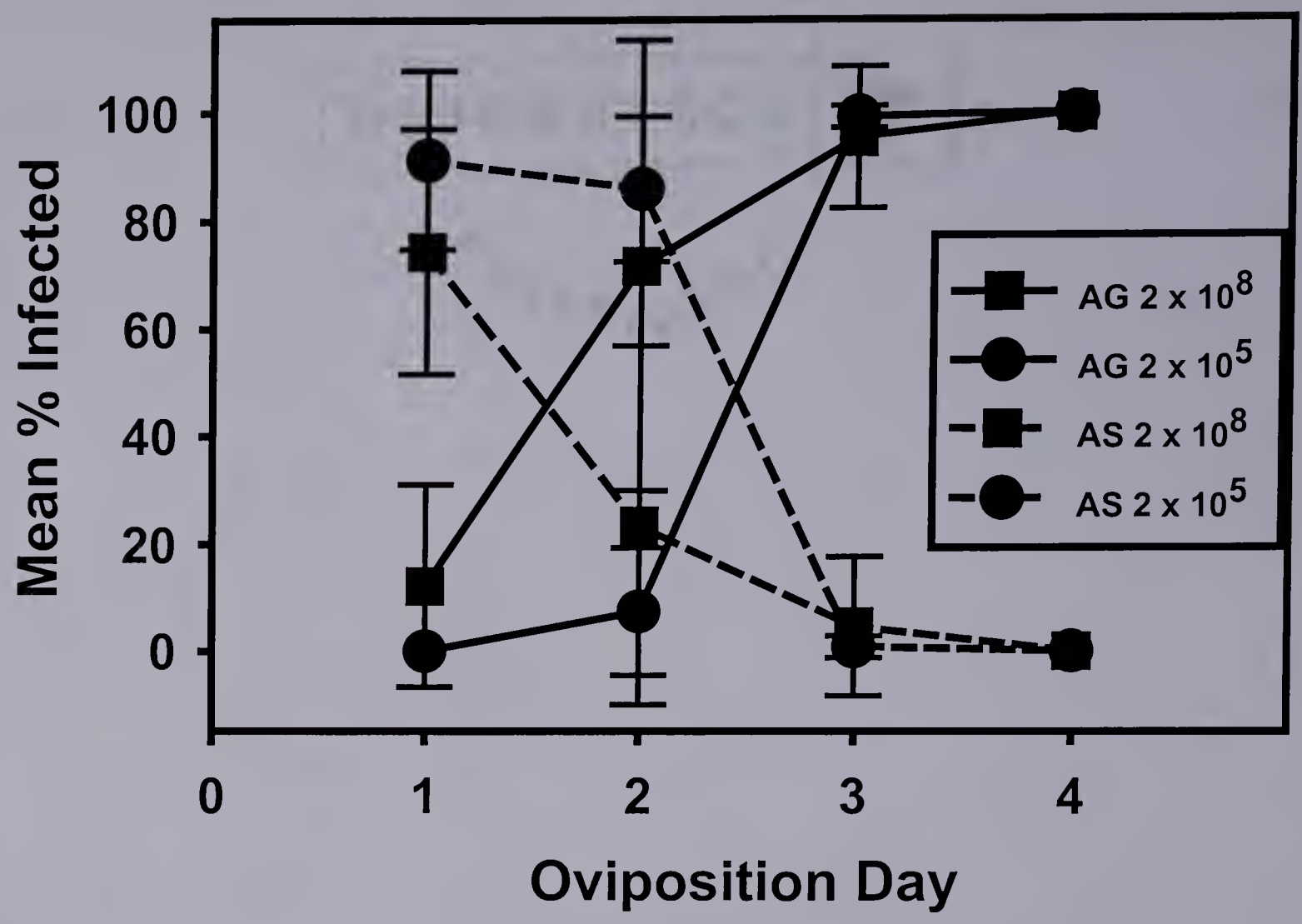

Mean % Infected
100
80
60
40
20
0
0
1
2
3
4
Oviposition Day
AG 2 x 10^8
AG 2 x 10^7
AG 2 x 10^6
AG 2 x 10^5
AS 2 x 10^8
AS 2 x 10^7
AS 2 x 10^6
AS 2 x 10^5

Figure 4.5. Agonadal (AG) and asymptomatic carrier (AS) progeny of parent females experimentally infected at the highest dose (2 x 10^8 $TCID_{50}$ units) and the lowest dose (2 x 10^5 $TCID_{50}$ units) of Hz-2V, demonstrating the correlation between virus dose and the relative percentage of agonadal and asymptomatic progeny. Asymptomatic carrier progeny are represented by broken lines in the figure, and agonadal progeny by solid lines.


Mean % Infected
100
80
60
40
20
0
0
1
2
3
4
Oviposition Day
AG 2 x 10^8
AG 2 x 10^5
AS 2 x 10^8
AS 2 x 10^5

CHAPTER 5

HZ-2V TRANSMISSION AND COMPREHENSIVE DISCUSSION

Introduction

In previous chapters (II & III), a description was provided about how the evolution of Hz-2V in *H. zea* has resulted in the ability of the virus to reprogram the development of male and female reproductive tissues into malformed, Y-shaped structures. These gross malformations result in sterile moths designated as agonadal. These virus-induced changes in development appear to favor productive virus replication in the malformed reproductive tissues while retaining the functionality of the structures necessary to initiate copulation and transmit Hz-2V to healthy mates.

Previous experiments conducted by Kingan et al. (1993) showed that only the lower portion of the male reproductive tract, specifically the cuticular segment of the ductus ejaculatorius simplex (DES), aedeagus, and endophallus, is essential for the successful initiation of copulation and transfer of reproductive fluids into the female moth. Although the ductus ejaculatorius and the primary segment of the DES are grossly malformed, and the testes are unfused and severely under-developed in agonadal male moths, these moths do retain the cuticular segment of the DES, the aedeagus, and the endophallus structures, which although slightly malformed, are apparently functional (chapter II). In addition, evidence was presented of Hz-2V replication in the cells of the malformed primary segment of the ductus ejaculatorius simplex and of an accumulation of virus particles in fluids within the lumen.

These observations suggest that sterile, agonadal male moths are capable of mating with and transmitting Hz-2V and any materials produced by the malformed DES,

ductus ejaculatorius duplex (DED), and vasa deferentia to female moths. During these matings, muscular contractions in the cuticular segment of the DES could force the material containing Hz-2V from the lumen of the malformed male reproductive tract through the endophallus and into the cervix bursa of a healthy female, possibly contaminating and even infecting her with virus. Subsequent matings between healthy male moths and these potentially newly contaminated females could produce infected progeny.

The malformed reproductive tissues of agonadal female moths produce very high titers of virus particles that are released into the lumen of the reproductive tract and ultimately exude from the vulva and coalesce into a viscous mass about the pair of reproductive openings (chapter III; Burand *et al.* 2004). This highly viscous material containing concentrated virus has been called a "waxy plug" (Raina and Adams 1995; Hamm *et al.* 1996). In addition, it has also been suggested that these agonadal females produce two to three times higher than normal levels of sex pheromone (Raina *et al.* 2000). These Hz-2V-induced structural and physiological changes in agonadal female moths seem ideally suited for the purpose of attracting males and then transmitting virus to them during attempts at copulation. A healthy male attempting to mate with an agonadal female could become contaminated during the initial act of securing the female with his genitalia and also during an attempt to insert the endophallus into the vulva of the infected female, bringing the endophallus into direct contact with the viral plug containing concentrated virus particles.

It has been reported in previous literature that agonadal females vigorously reject mates (Raina *et al.* 2000). It has also been reported previously that normal moths exhibit

similar behavior, in which females temporarily reject slow or inaccurate male advances by moving away a short distance until the male successfully clasps and holds her genitalia with his own (Callahan 1958). If a male makes several unsuccessful attempts to grab a female, she will often fly further away and resume calling. It is possible that the presence of the viral plug may make it more difficult for a male to successfully lock genitalia with an agonadal female, particularly if the viral plug is less viscous and covers the entire ventral region of the abdomen. Thus an agonadal female may in fact be receptive to mating if a male could successfully grasp and hold her.

Because the physiological and behavioral changes wrought by infection with Hz-2V in agonadal corn earworm moths clearly hint at a role in horizontal transmission during mating, experiments were conducted in which agonadal male and female moths were placed into two separate observation chambers and allowed to mate with healthy moths. Both agonadal male and female moths were observed to lock genitalia with the healthy moths, potentially contaminating them with virus, but the duration of these observed events was much shorter than is normal. When the previously healthy moths were then allowed to mate with new healthy moths, the pairs produced infected progeny, thus providing evidence that the agonadal moths were able to transmit virus to the healthy moths during copulatory events.

Materials and Methods

Source of Insects and Virus

Healthy, uninfected corn earworm larvae obtained from a USDA-ARS in Stoneville, MS, were used to start a laboratory colony of healthy *H. zea*. Insects were reared on artificial and maintained as outlined previously in chapter II.

Hz-2V for infecting female moths was prepared as described previously in chapter II and purified via sucrose gradient centrifugation (Burand and Lu 1997).

Injection of Adults and Selection of Infected Progeny Adults

Newly emerged, adult female moths were prepared and injected with high concentrations of Hz-2V as outlined previously in chapter II, in order to produce a high percentage of agonadal adults. Since agonadal male moths do not show any outward sign of their condition, male moths were selected from oviposition days in which all females were agonadal. The male moths were dissected after the experiment and their agonadal condition was verified. Agonadal females used in the second experiment were verified as agonadal by the presence of a viral plug, and then selected for the presence of viral plugs less than 1mm in diameter.

Mating Chambers

The glass observation chamber measured 24" long x 10" wide x 10" deep and was covered on three sides to prevent the intrusion of light. A screen top fitted with an egg-laying substrate (paper towel) was placed on top of the chamber, and a variable intensity fiber optics light source fitted with a Kodak #2380 deep red filter provided subdued light.

Several 1-oz cups containing 10% sucrose solution and with lids fitted with cotton wicks provided the moths with nourishment. Moths were observed under minimal light intensity for a period of two hours after the onset of the scotophase.

During the first experiment, five agonadal males were placed into the observation chamber and allowed to mass-mate for one scotophase with healthy females that had emerged prior to the previous scotophase. The potentially newly infected female moths were placed into individual mating chambers with healthy male moths and allowed to mate. Eggs resulting from these matings were collected daily and reared to adults as described for the colony.

For the second experiment, five agonadal females selected for viral plugs as described above were placed into the observation chamber and allowed to mass-mate for one scotophase with ten healthy males that had emerged prior to the previous scotophase. The potentially newly infected males were placed into individual mating chambers with healthy females and allowed to mate. Eggs resulting from these matings were collected daily and reared to adults as described for the colony.

DNA Extraction, PCR Amplification of Viral DNA Sequences, Detection of a Viral DNA Sequence by Slot-blotting, Ethidium Bromide Gel Electrophoresis

As described in the materials and methods of chapter IV, DNA was extracted from the reproductive tissues of adult moths by a boiling lysis method. Viral DNA used as template for PCR reactions was extracted from purified virus using 1% SDS in TE containing 1 mg/ml Protease K as outlined by Burand and Lu (1997). Two sets of primers were used to amplify Hz-2V genomic DNA to prepare a probe for use in slot-blot analysis of insect reproductive tissues. DNA extracted from reproductive tissues was

analyzed by slot blotting, and the size of the DNA fragment was verified the same as viral DNA by staining the DNA with EtBr and running on a 0.8% agarose gel also as described in chapter IV.

Results

The normal mating behavior of corn earworm moths is summarized here as described by Callahan (1958) for comparison to observed mating behavior of agonadal moths. During the second scotophase after emergence as an adult, the healthy female moth begins calling by lifting and rapidly vibrating their wings while extruding the ovipositor and releasing sex pheromones. The healthy male responds to this calling by approaching the female, twisting his abdomen at an angle that brings his abdomen closer to hers, and then extending his claspers in an attempt to lock the female's genitalia with his own. If successful, the pair begins copulating, and the male eventually transfers a spermatophore and male reproductive secretions into the bursa copulatrix of the female, a process that normally lasts between one and two hours. If the male is too slow or inaccurate in his attempted grab, the female often becomes mildly agitated and moves a slight distance away. The male may make several attempts before either he successfully locks with the female, or she becomes very agitated and flies away from him.

After the mated pair separates, the male often attempts to mate with another calling female. The mated female, however, ceases calling and her level of sex pheromone drops rapidly due to the transfer of male-derived anti-calling factors and a pheromonostatic peptide (PSP-1), respectively (Kingan *et al.* 1995). A mated female does not normally mate again during the current scotophase, but will become receptive again and call during the next scotophase.

Agonadal male moths were observed to respond normally to the calling of healthy female moths, approaching and twisting their abdomens, attempting to use their genitalia to clasp those of the females. During the two-hour observation period, some agonadal

males were observed to successfully lock genitalia with females, but separated after less than three minutes. After separating, the males rested briefly before pursuing another calling female and attempting to mate again.

In a separate chamber, agonadal females began calling about one hour after the beginning of the scotophase, lifting and vibrating their wings while extruding their ovipositors, as do healthy females. Healthy male moths were attracted to the agonadal females and were able to successfully approach and grab the females' genitalia with their own. However, these observed matings only lasted two to three minutes, after which the partners separated. The males often rested briefly before attempting to find another mate.

Progeny resulting from healthy females mated with agonadal males and subsequently with healthy males produced asymptomatic carrier progeny by the first oviposition day and agonadal progeny by the third oviposition day following the final mating (Table 1). Of the total progeny resulting from these matings, approximately 11% exhibited the agonadal condition, 76% were asymptomatic carriers of Hz-2V, and the remaining 13% were healthy. About 87% of the total progeny resulting from these mating events were apparently infected with Hz-2V.

Matings involving healthy males first mated to agonadal females and subsequently to healthy females resulted in approximately 92% of the total progeny arising from eggs laid on the first two oviposition days following the last mating event being asymptomatic carriers of Hz-2V (Table 2). The newly-contaminated female moths did not produce any viable eggs after the second oviposition day, nor did they produce any agonadal progeny.

Comprehensive Discussion

The evolution of Hz-2V in its insect host, *Helicoverpa zea*, has resulted in the ability of the virus to exist in a persistent state and yet be induced into productive replication later on in the development of the insect. In addition, the virus may cause either agonadal or asymptomatic carrier infections in infected moths, likely depending on the amount of virus present in the developing larva (chapter IV). Low-dose infection with Hz-2V in these moths can lead to the development of apparently healthy, fertile asymptomatic carriers in which the virus persists and is likely induced into productive replication during the pharate adult or adult life stage. High dose Hz-2V infections result in sterile, agonadal moths, in which the reproductive tissues are grossly malformed into Y-shaped structures favorable for productive virus replication in males and females (chapters II and III). In addition, infection with Hz-2V in these agonadal moths leads to changes in the physiology and the mating behavior of males and females, ultimately enabling agonadal moths to transmit the virus to healthy mates during attempts at copulation.

In agonadal male moths, the virus-induced malformations result in reproductive tissues in which the accessory glands are absent, the primary segment of the DES and also the DED are dramatically reduced, the testes are under-developed and unfused, and the vasa deferentia are enlarged to form the upper lobes of the Y-shaped structure. The cuticular segment of the DES, the aedeagus, the endophallus, and the genitalia appear to be functional, although slightly malformed. Virus replication was found to occur in the malformed primary simplex, and a white, viscous liquid isolated from the lumen of the malformed DES and DED strongly resembled that found in oviducts in female moths and

was shown to be comprised of a concentrated mass of virus particles in a granular, darkly stained material (chapter II).

Kingan *et al.* (1993) conducted experiments in which the testes, vasa deferentia, duplex, and accessory glands were removed from male moths and found that these moths were still able to mate with and transfer reproductive materials to the bursae of female moths. Based on these data, all of the male reproductive structures necessary for initiating copulation and transferring reproductive fluids to the female moth are present and appear to be functional in agonadal male moths. Healthy females allowed to mate with agonadal males and subsequently to healthy males produced infected progeny, strongly indicating that these male structures are functional, and that Hz-2V present in the lumen of the DES and DED can be transmitted from an agonadal male to a female moth during attempts to copulate (Table 1).

The malformed, Y-shaped structure in agonadal females is mainly comprised of enlarged oviduct tissues, which develop several layers of epithelial cells favorable for virus replication and lack the cuticular lining bordering the lumina (chapter III). Hz-2V replicates to very high titers in these reproductive tissues and is released into the lumina when the cells lyse, eventually concentrating in the malformed bursa copulatrix. Ultimately, the concentrated virus mass exudes out of the vulva and forms a viscous viral plug about the reproductive openings (chapter III; Burand *et al.* 2004). In addition, these agonadal females may produce higher levels of sex pheromone than do normal females (Raina *et al.* 2000), possibly enhancing their ability to attract mates. Based on the results presented in Table 2, in which infected progeny result from matings begun with agonadal

female moths, healthy male moths attracted to these infected females become contaminated with Hz-2V while attempting to mate.

Agonadal males and females were able to successfully initiate copulation with healthy mates, and although the duration of observed matings was much shorter than normal, the physical contact lasted long enough for the agonadal moths to transmit Hz-2V to healthy mates, which were then able to transmit the virus to progeny resulting from subsequent matings with other healthy, fertile moths (Tables 1 & 2). Interestingly, females mated to agonadal males rested briefly after the pairs separated and then resumed calling, indicating that they were still sexually receptive.

It is possible that the behavior reported by Raina et al. (2000) in which the agonadal females aggressively avoided mating with males may have been the same as the normal mating behavior of female *H. zea* as described by Callahan (1958), but appeared different due to the males' inability to successfully clasp and hold the females' genitalia. As a result of inadequate male advances, the females would reject the males and move away. The fact that females were rejecting mates implies that the agonadal females were able to attract males. The size and consistency of the viral plug over the vulva of agonadal female moths varies considerably, ranging from almost no visible plug to plugs large enough to completely encompass both reproductive openings. It is possible that a larger or less viscous viral plug on the tip of the abdomen of an agonadal female would make it more difficult for a male to successfully grab and lock genitalia with the female, leading her to eventually reject his advances and move a significant distance away from him. To avoid this potential problem in these experiments, agonadal females with small viral plugs less than 1 mm in diameter were selected for these mating experiments.

These data represent evidence that agonadal adult male and female corn earworms infected with Hz-2V are effective vectors of the virus. Infection with Hz-2V alters the physiology and mating behavior of these agonadal moths and enables them to successfully mate with and transmit the virus to healthy mates, which were able to transmit the virus to up to 92% of the progeny resulting from subsequent matings with healthy, fertile moths. Female moths normally mate only once during a given scotophase and lose sexual receptivity for the rest of that scotophase due to male-derived anti-calling factors including the spermatophore, and a pheromonostatic peptide (PSP-1) secreted by the reproductive system of normal male moths and transmitted to females during copulation (Kingan *et al.* 1995). Yet agonadal females continue to call and attract mates after mating with healthy male moths, possibly enabling these females to contaminate several males during each scotophase. Because male corn earworms are promiscuous, both agonadal males and any males that may have become contaminated with Hz-2V after attempting to mate with agonadal females could potentially mate with and infect or contaminate multiple healthy females during any given scotophase, enabling the spread of Hz-2V throughout the population.

Based on the correlation between dose and the outcome of infection presented in the dose-response experiment (chapter IV), the low doses of virus transmitted to healthy moths during mating attempts begun with agonadal moths and later performed with contaminated moths would produce primarily asymptomatic carrier progeny, favoring virus persistence within a population. However, it is apparent that agonadal male moths are more efficient vectors of Hz-2V, transmitting larger amounts of virus along with reproductive fluids directly into healthy female moths, which are then able to transmit

higher doses of Hz-2V to progeny resulting from subsequent matings with fertile males. This is indicated by the data from the agonadal male mating experiment, in which agonadal progeny was produced, suggesting higher doses of virus were transmitted to these progeny insects.

This method of virus transmission to healthy mates during copulation minimizes problems associated with the virus remaining viable while being exposed to external factors in the environment, such as sunlight, ultraviolet light and desiccation. Insect baculoviruses have evolved the formation of crystal occlusion bodies to keep the virus viable until ingested by a new host (Miller and Ball 1998), whereas Hz-2V forms round, virus-filled vesicle structures (Burand *et al.* 2004). The virus in these vesicles would not remain viable for long if exposed to UV irradiation or desiccation. Instead, Hz-2V has evolved a more direct method of transmission relying on the intimate contact between agonadal and healthy moths, thus limiting the exposure of the virus to environmental factors that could decrease its viability.

In evolving the pattern of infection seen in the progeny of infected moths (chapter IV), in which healthy progeny are produced initially, the virus ensures that the host population survives, maintaining a ready supply of new, susceptible host insects. The production of healthy, uninfected progeny is followed by Hz-2V-infected progeny that develop into asymptomatic carriers, which enable the virus to persist in the population by mating and transmitting the virus transovarially to a significant number of resulting progeny. On the latter oviposition days, the eggs laid by infected moths develop into agonadal moths that have the potential to spread Hz-2V throughout the population by

transmitting the virus during copulation as described here. By employing this dual infection strategy, Hz-2V ensures both its persistence and its transmission to new hosts in a population of *H. zea*.

Table 5.1. The percentage of progeny moths infected with Hz-2V transmitted initially from agonadal males. "Asymptomatic carriers" are abbreviated as "Asymp". Percent infected was calculated using the sum of the asymptomatic carrier moths and the agonadal moths.

Oviposition day	Healthy	Asymp.	Agonadal	% Infected
1	1	11	0	91.7
2	4	17	0	81
3	2	14	6	90.9
Total progeny	7	42	6	87.3

Table 5.2. The percentage of progeny moths infected with Hz-2V resulting from matings begun with agonadal females. "Asymptomatic carriers" are abbreviated as "Asymp". Percent infected was calculated using the sum of the asymptomatic carrier moths and the agonadal moths.

Oviposition day	Healthy	Asymp.	Agonadal	% Infected
1	4	30	0	88.2
2	1	26	0	96.3
Total progeny	5	56	0	91.8

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