Effect of Diet on Adult House Fly (Diptera: Muscidae) Injected With the Salivary Gland Hypertrophy Virus (MdSGHV)

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Subject Editor: Michael Strand
Received 1 January 2018; Editorial decision 15 April 2018

Abstract
Research to date on the salivary gland hypertrophy virus (SGHV) in three species of flies has focused on adult flies having access to and taking a proteinaceous diet. Since many studies have shown that diet affects viral infection in numerous organisms, this study examined the effect of a protein-free diet on the effect of the SGHV virus in adult house flies, Musca domestica, L. Adults infected with the virus, and maintained on a sugar diet only, showed salivary glands with a blue rather than a grayish color and mild hypertrophy compared with protein-fed flies. It was possible to retrieve the virus from these glands and successfully infect noninfected flies. When injected at various ages, female flies fed only sugar showed that regardless of age, sugar-fed flies still became infected and showed the pathology of the glands. In addition, electron microscope studies revealed at the ultrastructural level that there was no difference between viral replication in cells from salivary glands of adults fed a proteinaceous-free diet and those feeding on protein.

Key words: corpus allatum, juvenile hormone, nutrition-immunity

Introduction
Salivary gland hypertrophy viruses (SGHVs) of the family Hytrosaviridae have been reported in three species of flies: Glossina sp. (Jaenson 1978), the house fly (HF), Musca domestica (Coler et al. 1993), and the narcissus bulb fly, Merodon equestris (Amargier 1979). These viruses replicate principally in the salivary glands of the flies causing hypertrophy and often resulting in infected flies being infertile. In Glossina sp., it is assumed that previous reports only focused on flies that already received a proteinaceous bloodmeal (Kokwaro et al. 1990). No information has been reported on M. equestris, but it is also assumed that specimens examined received sufficient amino acids from either pollen and/ or nectar or the flies are autogenous. Weigel (1926) reported adults able to produce eggs only on honey, thus suggesting they might be autogenous. HF adults, however, are mainly anautogenous (Adams 1970) (i.e., they need a protein meal in order to develop not only their ovaries (Adams and Nelson 1990) but also to initiate mating (Adams and Hintz 1969). Consequently, on a diet of sugar alone, most anautogenous flies cannot develop their ovaries and usually fail to mate (Yin and Stoffolano 1990). The importance of diet on viral infection in several animal systems (Sprunt and Flanigan 1956, Beck and Levander 2000), including the honey bee (DeGrandi-Hoffman and Chen 2015) and Drosophila (Arnold et al. 2013), emphasize the importance of proper nutrition or lack of it on viral replication. After reading these papers and the review by Katona and Katona-Apte (2008), it raised the question for this study concerning the role of diet (i.e., sugar alone) on virus-infected, adult HF. Basically, organisms with poor nutrition generally have reduced immunity to viral infection. If this is true, what effect does a protein-free diet have on replication of the HF SGHV (MdSGHV) in its host, and is viral infectivity to other adult HF compromised? Based on these reports and the report of Lietze et al. (2007) where all of their experiments were based on adult HF having protein in the diet, it was decided to examine the fate and effect of the virus on adult HFs receiving only a sugar and water diet (i.e., a protein-deficient diet) prior to and following injection of the HF salivary gland virus. To our surprise, initial dissections revealed flies with darker blue rather than the usual grayish-blue salivary glands (Coler et al.
Thus, we wondered if the virus from the salivary glands of these protein-deprived flies was infectious when injected into other noninfected hosts and would the hypertrophied pathology of the glands be evident at the transmission electron microscope (TEM) level. Objectives of this study were to characterize these blue salivary glands using TEM, compare measurements and micrographs of the salivary glands of sugar-fed flies with protein-fed flies (i.e., from other TEM studies), and examine the infectivity of the virus from salivary glands of sugar-fed flies into noninfected HF fed a protein diet.

**Materials and Methods**

Obtaining and Maintaining the Flies and MdSGHV

HF pupae and MdSGHV were obtained from Dr. C. Geden, USDA-ARS, Gainesville, FL, who raised the flies according to that of Hogsette (1992). MdSGHV was received as one infected fly equivalent in 0.1-ml sterile water. For injections, 100 ml of phosphate buffered saline (PBS) was added to 0.1 ml/injected fly equivalent prior to injection into flies. Adults were kept in 20 × 20 × 20 cm aluminum-screened cages in a fly rearing room maintained at 26°C, 40% RH, and 16L:8D photoperiod (Stoffolano 1974). Flies were given a diet of granulated sugar only or a 50:50 mix of granulated sugar and whole milk powder (Stoffolano 1993). MdSGHV was received as one infected fly equivalent in 0.1-ml sterile water. For injections, 100 ml of phosphate buffered saline (PBS) was added to 0.1 ml/injected fly equivalent prior to injection into flies. Adults were kept in 20 × 20 × 20 cm aluminum-screened cages in a fly rearing room maintained at 26°C, 40% RH, and 16L:8D photoperiod (Stoffolano 1974). Flies were given a diet of granulated sugar only or/and powdered whole milk and granulated sugar in a 50:50 mix in 540-ml clear plastic cups. Water was provided in a 540-ml plastic cup with Absorbal cotton wicks through two holes in the lid to prevent drowning. To maintain MdSGHV, adult HFs were injected with the virus as described above and dissected 7 d postinjection. Infected salivary glands were put into 1.5-ml autoclaved centrifuge tubes with 500 µl of PBS in each tube. Each tube contained a pair of salivary glands for each fly and was homogenized with a manual pestle. The homogenized tissue was drawn up into an 18G ½ needle attached to a B-D single use PlastiPak 3 cc syringe with Luer-Lok tip. A 0.45-µm Acrodisc syringe filter was added to the syringe, and inoculum was added to 1.5-ml centrifuge tubes with the addition of PBS for a final volume of 50 µl. Inoculum was stored in a −80°C freezer.

Injection of MdSGHV

Adults were cold immobilized for 2 min at −20°C before being injected. Injections were performed using a B-D Safety-Lok, 1-ml 29G1/2 insulin syringe (0.3 × 13 mm) with an ULTRA-FINE needle, attached to a foot-pedal–activated kdScientific syringe pump. Using BioQuip featherweight forceps, the fly was placed on the syringe needle, while 2.5 µl of the MdSGHV sample diluted 1:1000 in PBS was injected into the side of the mesothorax. Flies used in each treatment were infected with the same virus sample prepared in the same manner. After injection, flies were put into clean 20 × 20 × 20 cm aluminum cages with water and either granulated sugar only or a 50:50 mix of granulated sugar and whole milk powder. After injection, caged flies were kept in a Percival Intellus environmental chamber set to 26°C, 40% RH, and 16L:8D photoperiod (Stoffolano 1974) for 7 d at which time infection/pathology was assessed by dissection.

Obtaining the Viral Inoculum From the Salivary Glands of Sugar-Fed Flies

Viral-infected flies were dissected, and the infected salivary glands were put into 1.5-ml centrifuge tubes with 0.5 ml of PBS and homogenized with a hand pestle. The inoculum was filtered through a 0.45-µm syringe filter then stored at −80°C freezer until used to verify infectivity and for PCR analysis. Flies injected with PBS alone served as controls.

Dissection of Salivary Glands and Imaging

Flies were put into a plastic cup and placed in a −80°C freezer for 4 min, then individually put on a dissecting dish using Ento-pins to secure them under a Bausch & Lomb dissecting microscope. Approximately, 300 µl of PBS was used to flood the abdomen of the fly, and stainless steel no. 5 forceps used to dissect the abdomen and remove the salivary glands. To best remove the glands, the salivary glands were grasped in the neck region by the common duct of the salivary glands. Each pair of glands was put into 1.5-ml microcentrifuge tubes with 50 µl of PBS. Glands from infected flies used for measurements (N = 46 for both sugar-fed and N = 44 for protein-fed flies) were put on a glass slide in PBS and measured on an Olympus Phase microscope with an ocular micrometer. Two measurements were taken: one consisted of measuring the terminal bulb of the salivary gland (Fig. 1, DM), while the other was to measure the narrower width, which was just prior to the bulb (Fig. 1, asterisk). Images were taken with an AxioCam ERC5s with Zen imaging program.

**PCR Protocol**

MdSGHV infection of salivary glands from protein-deprived flies was confirmed by PCR analysis with two different primer sets. DNA from infected salivary glands of sugar-fed flies (N = 10) was purified with Qiagen DNeasy Blood & Tissue Kit according to the manufacturer’s protocol. PCR cycling conditions were at 94°C for 5 min for initial template denaturation, followed by 35 cycles of 94°C for 45 s, 45.2°C for 45 s, 72°C for 1 min, with a final step of 72°C for 7 min. Two primer sets were used, a degenerate p74 primer set and a primer specific for MdSGHV ORF106 (Abd-Alla et al. 2009). PCR products were analyzed on 1% agarose gels (results not included).

![Fig. 1. SEM of the distal section of the salivary gland of a protein-fed, adult *Musca domestica* showing where the measurements for Figs. 2 and 3 were taken. DM is the enlarged distal bulbous part. *The other measurements were taken just posterior to this area (i.e., at the narrower site). Scale bar = 100.0 µm.](image-url)
TEM and SEM of Salivary Glands
TEM sections were prepared and viewed according to Guerra et al. (2015). For scanning electron microscopy (SEM), flies were dissected in PBS, the salivary glands were carefully removed and placed in 80% ethanol until critical point dried by use of liquid CO₂. Glands were then mounted on stubs, sputter coated with 15-nm gold and examined in an FEI Quanta 200 SEM (FEI Company, Hillsboro, OR), at 10 kV at the electron microscopy facility at Mt. Holyoke College, South Hadley, MA.

Effect of Age on Susceptibility of Sugar-Fed Flies
In order to examine the effect of diet on the susceptibility of flies at a particular day postemergence, a cage of male and female flies (N = 110) was prepared, and flies given only granulated sugar until days 14–39 when female flies (all males in the cohort had died by day 14) were injected with the virus as outlined above. Seven days following injection at days 14–39, all remaining flies were dissected to determine susceptibility based on salivary gland hypertrophy.

Statistics
Using Excel, the t-test of two samples, assuming unequal variances performed for the width of the narrow region and terminal bulbous region of salivary glands of viral-infected, adult HF, was performed to determine statistical significance.

Results
Effect of Virus on the Size of the Salivary Gland of Sugar-Fed Flies
Virus-injected, adult HF of both sexes had smaller salivary glands when the fly diet was restricted to only sugar (N = 46) compared with flies fed protein and sugar (N = 44). Within 7 d after injection with virus prepared from protein-fed flies, all infected, sugar-fed flies showed the dark blue color symptoms of the SGHV infection and mild hypertrophy. HF that was provided a protein/sugar diet had salivary glands on average 23.75% larger than flies fed only sugar (Figs. 2, 3). A t-test of two samples, assuming unequal variances, was performed for either the narrower width or bulb width of the viral-infected, adult HF’s salivary glands. The t Stat was 5.57, and the t critical was 1.66. The t Stat is larger than the t critical value, thus the results are statistically significant.

Effect of a Sugar-Fed Diet on Competency of the Virus to Infect Other Flies
Flies fed a restricted diet of sugar-only were injected with SGHV and dissected 7 d postinjection. Salivary glands were retrieved and made into an inoculum. The inoculum was injected into noninfected, sugar-fed-only flies, to test the virulence of virus from flies with a restricted diet. All flies injected (N = 23) were dissected 7 d postinjection. These flies all showed mild hypertrophy with the blue color symptoms of SGHV and were PCR positive for the presence of virus.

Effect of Age on Susceptibility of Sugar-Fed Flies
Of the 110 original flies, only females survived and represented a 72% survival of flies given only sugar. Number of flies dissected at the various days posteclosion varied with a minimum of one fly each at 37 and 39 d. Otherwise, the surviving fly number at various days varied from 3 to 15. Dissection of the surviving 80 females 7 d postinjection revealed that 100% of the sugar-fed females showed pathological symptoms typical of SGHV infection indicating their ability to support viral replication.

Effect of Diet on the Ultrastructure of the Salivary Glands of Sugar-Fed, Viral Injected Flies
TEM of the salivary glands of only sugar-fed flies shows ultrastructural evidence of viral replication reminiscent of that shown for protein-fed flies, in other studies, including the assembly of nucleocapsids in the nucleus and bundles of enveloped virus particles in

Fig. 2. Influence of diet on the salivary glands of viral-infected adult house flies of both sexes measured as width at the narrow part of the gland (see * in Fig. 1). The narrower width was measured just prior to the terminal bulb of the gland. PS = 350.4 and S = 276.0; P ≤ 0.05.

Fig. 3. Effect of diet on the distal bulb (see Fig. 1, DM) of the salivary gland of viral-infected, adult house flies of both sexes.
the cytoplasm of infected cells (Fig. 4) (Coler et al. 1993, Geden et al. 2008). While clusters of nucleocapsids were found associated with the virogenic stroma, individual particles where observed in the periphery of the nucleus often aligned with and apparently exiting the nuclear membrane.

**Discussion**

The salivary glands of insects are extremely important in the replication cycle and vectoring of a variety of viral pathogens. Thus, the adult HF, and its associated virus, is an excellent model system to study insect vectoring of viruses whereby the mechanisms of infection and transmission still need to be clarified (Kariithi et al. 2017a). The ability of the virus to take over development of the ovaries and prevent egg development, plus the impact the virus has on preventing mating could lead to development of novel ways to control this world-wide pest in the future.

Reports have shown that diet affects salivary gland gene expression in mice (Simon et al. 2015), plus environmental factors (i.e., including diet) are believed to be involved in gene expression of saliva production in sand flies (Coutinho-Abreu and Ramalho-Ortigao 2011). As important as the HF is as a vector of pathogens to both humans and animals, very little research has focused specifically on the salivary glands of this insect. One of the objectives of this study was to examine the effect of a nonprotein diet (i.e., only sugar) on both the salivary gland development in viral-infected HF and to determine whether the virus replication proceeds in these dietary-restricted flies culminating in infectious virus particles. First, one must ask “What are the hormonal conditions of flies deprived of protein?” The review by Wyatt et al. (1996) on the role of juvenile hormone (JH) on adult organs, however, fails to mention salivary glands, while numerous studies show that without JH, the accessory reproductive glands (ARGs) of many adult flies fail to develop (Yin and Stoffolano 1997). If the ARGs are affected by diet and lack of JH, why would not the salivary glands respond in the same way since both are important in fly maturation? This study represents the first report demonstrating that salivary gland development in adult HF is dependent on a proteinaceous diet. Without sufficient protein in their diet, two fly species fail to produce eggs and to mate—both processes of which are dependent on JH (HF—Adams and Hintz 1969 and Phormia regina—Yin et al. 1997, 1999). Diet has also been shown to influence egg development in both species (Adams and Nelson 1990, Yin and Stoffolano 1990). Initially, Kariithi et al. (2017a) noted that the presence and effect of the MdSGHV in the corpus allatum/corpus cardia- cum complex help explains the low levels of sesquiterpenoids (i.e., JH acyclic sesquiterpenoid) in viremic flies. Later, Kariithi et al. (2017b), however, correctly report that virus in the corpus allatum (i.e., where the sesquiterpenoid is present in producing JH) can have an effect on hormone production. If they also observed virus in the corpus cardiacum, it is possible that other hormones or neuropeptides are affected. Consequently, as shown for the above-mentioned two fly species, a protein-deficient diet results in no mating or egg development, both of which are due to insufficient JH levels and lack of a protein-based diet. The blue color of the salivary glands in SGHV-infected sugar-only-fed flies, in contrast to the grayish color reported by Coler et al. (1993), could be due to the Tyndall Effect (TE). TE occurs where the longer light wavelengths are transmitted while the shorter light wavelengths are reflected due to scattering of the light. Because the salivary glands are smaller, but still mildly hypertrophied, in sugar-fed flies (i.e., they may have a greater concentration/unit volume of viral particles than glands that show maximum hypertrophy, thus the TE would be more observable). This enhanced TE could also be due to a more efficient virus production in sugar-fed flies where the hormonal cascade leading to egg development is uncoupled from SGHV replication. A protein-free diet for adult, virus-infected flies did not dramatically impact the ability of the virus from the smaller, mildly hypertrophied salivary glands to infect noninfected flies. Also, of the 80 sugar-fed, only females that survived to old age (i.e., from 15 to 39 d) when injected at different ages all became infected and showed evidence of SGHV pathology. This demonstrates that even though these flies were not only older, but if fed a protein diet would have undergone two ovarian cycles, they were still able to become infected. Because a protein-free diet prevents flies from producing eggs (i.e., HF is anautogenous), it was not possible to determine whether lack of ovaries was due to the protein-free diet or the virus. Diet did, however, affect the size of the salivary glands. At the same time, flies having a protein-restricted diet had significantly smaller salivary glands but showed the typical viral pathology at the TEM level. Examination and comparison of the ultrastructural results of only sugar-fed, adult HF when compared with previously published TEMs (Coler et al. 1993, Geden et al. 2008) showed little structural differences. It is unlikely, however, that diet restriction in the wild exist, and studies need to be conducted to see whether virus infection affects food choice as was shown by Van Den Abbeele et al. (2010) where the parasite Trypanosoma brucei modified the salivary composition of tsetse and altering of feeding behavior.

**Conclusion**

Diet was shown to have physiological effects on the fly’s salivary glands when it comes to size. House flies with protein in their diet had glands 23.75% larger than those only fed sugar suggesting that the lack of protein in the diet influences not only JH production but also the size of the glands compared with protein-fed flies (Yin and Stoffolano 1990). Even with the lack of size increase of salivary glands in sugar-fed, viral-infected flies, virus replication in these
glands appeared normal and was infectious when injected into non-infected flies fed only sugar.

Acknowledgments

Appreciation to Dr. C. Geden, USDA in Gainesville, FL, for supplying the pupae to start our own colony. Thanks to Dr. R. Wick for the use of his microscope for making measurements of the salivary glands. Thanks to A. Frappier for providing the data on the age-effect on infectivity when flies were injected with the virus at various days postemergence. This study is based upon research supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, the Massachusetts Agricultural Experiment Station (MAES) and the Stockbridge School of Agriculture at the University of Massachusetts, Amherst, under project number MAS00448 to J.G.S and MAES project number MAS00470 to J.P.B. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA or NIFA. Thanks to M. Rice, former director of the Science Center Microscopy Facility at Mt. Holyoke College, South Hadley, for preparing the glands for SEM and Mya Thandar for taking the SEM photos. Salivary glands of sugar-fed-only flies were embedded for sectioning by Blanca Carabajal Gonzalez also at Mt. Holyoke College. All other TEM work was done in Italy.

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