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Effectiveness of Pathogen-Specific Passive Antibodies to Mitigate Infectious Diseases in Apis Mellifera

Tanner Nordseth
University of South Dakota

EFFECTIVENESS OF PATHOGEN-SPECIFIC PASSIVE ANTIBODIES TO MITIGATE INFECTIOUS DISEASES IN *APIS MELLIFERA*

by

Tanner Nordseth

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The members of the Honors Thesis Committee appointed

to examine the thesis of Tanner Nordseth

find it satisfactory and recommend that it be accepted.

_____

Dr. Benjamin Hause
Assistant Professor of Veterinary and Biomedical Sciences
South Dakota State University
Director of the Committee

_____

Mr. Jonathan Mahlberg
Vice President of Manufacturing
Cambridge Technologies

_____

Dr. Victor Huber
Associate Professor of Basic Biomedical Sciences
University of South Dakota

ABSTRACT

Effectiveness of Pathogen-Specific Passive Antibodies to Mitigate Infectious Diseases in *Apis Mellifera*

Tanner Nordseth

Director: Benjamin Hause, Ph.D.

Honeybees (*Apis mellifera)* are widely recognized as a vital part of the global ecosystem and the world's food supply due to their pivotal role in the pollination of both natural and agricultural flora. Colony Collapse Disorder (CCD) is an emerging phenomenon characterized by a colony's worker bees deserting the hive and leaving the queen behind. This usually results in colony failure. CCD is a multifactorial issue, with many environmental stressors and pathogens playing a role. Deformed Wing Virus (DWV) has been identified as a leading cause in this phenomenon. *Paenibacillus larvae (P. larvae)* is another lethal pathogen that is responsible for American Foulbrood Disease and can also ultimately lead to colony collapse. Honeybees have ineffective innate immunity against these pathogens and lack an adaptive immune system altogether.

In the research being presented, novel passive antibody therapy was used to treat DWV and *P. larvae*. To develop this therapeutic, white leghorn hens, which are known to confer passive immunity to the yolks of their eggs in the form of Immunoglobulin Y (IgY), were vaccinated against one of these antigens. The egg yolks were harvested, diluted, and purified to produce concentrated IgY specific to either DWV or *P. larvae*. This purified IgY was added to the larval diet and orally administered to 1st instar larvae

in a challenge study to test its ability to protect against either DWV or *P. larvae* challenge. Experimental results revealed that among larvae challenged with DWV, mortality dropped from 79% to just 29% when a 1:100 dilution of DWV-specific IgY was administered. Likewise, larvae challenged with *P. larvae* saw mortality drop from 100% to only 17% when treated with a 1:100 dilution of *P. larvae*-specific IgY. The mortality trends in both models were supported by corresponding molecular data in the form of either RT-PCR or CFU/mL data. These results suggest that pathogen-specific IgY may provide honeybee larvae with approximately 50% protection against lethal DWV disease and 80% protection against lethal *P. larvae* disease – figures that would significantly reduce CCD. This technology could have a profound impact on the future of beekeeping – an industry that is key to avoiding a global food sustainability crisis.

KEYWORDS: Deformed Wing Virus (DWV), Colony Collapse Disorder (CCD), Immunoglobulin Y (IgY), honeybees, *Apis mellifera, Paenibacillus larvae* (*P. larvae)*

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CHAPTER ONE

Introduction

Agricultural and Economic Significance of Apis Mellifera

Originating in East Asia, the western honeybee *(Apis mellifera)* is widely renowned as perhaps the single most important organism for maintaining agricultural sustainability and biodiversity among flowering plants.[1,2] Bees are responsible for the pollination of more than 400 commercially grown agricultural plants and approximately 1/6 of the world's natural flowering plants.[3] Unlike other pollinators, *A. mellifera* is considered a "generalist forager" because it visits such a wide array of plants.[4] Honeybee colonies are rented around the globe for the production of hive products (i.e. honey) and pollination services (90% of commercial pollination occurs via *A. mellifera)*, making this one of the most economically important insects.[2,5] Crops such as almonds are entirely dependent on *A. mellifera* for pollination; blueberries and cherries are approximately 90% dependent; and nearly every other fruit, nut, vegetable, melon, and field crop produced in the United States is at least partially dependent upon the honeybee.[6,7] Today, there are approximately 2.7 million *A. mellifera* colonies in the United States, with over 1.8 million in California alone to support the state's almond industry.[7] In recent decades, honeybees are also steadily increasing their value contributed to the agricultural economy, with their pollination services and honey products contributing an estimated $9.3 billion in 1987, $14.6 billion in 2000, and $20 billion in 2020 to U.S. crop production.[6,7]

Colony Collapse Disorder

In recent years, *A. mellifera* has become increasingly afflicted by Colony Collapse Disorder (CCD), a multifactorial phenomenon that induces the swift demise of an entire colony. CCD is best characterized by the rapid disappearance of a colony's adult worker bees, leaving behind a seemingly healthy colony containing food, brood, and a queen.[8] As the honeybee population continues to dwindle, it has been surmised that malnutrition from a decrease in flower diversity and the increase in pesticide usage, especially neonicotinoids, are two likely contributors.[9,10] There is also evidence suggesting that the rise in global CO_2 levels may be causing fundamental alterations in the physiology of pollen-producing plants that diminish the pollen's protein content and create nutritional deficits for *A. mellifera*.[11] There is ongoing debate regarding which of these factors are most problematic and what steps should be taken to protect *A. mellifera.* While each of these factors likely creates strain on the honeybee population, a pathogen known as Deformed Wing Virus (DWV) is widely considered to be the predominant stressor contributing to CCD.

Overview of Deformed Wing Virus

DWV is a critical honeybee pathogen that has captured the attention of apiculturists in recent years. The discovery of DWV began with the isolation of Egypt Bee Virus, a virus now known to be related to DWV that was isolated from asymptomatic adult worker bees in Egypt in 1977.[12] This discovery inspired further study of honeybee virology, and in 1982 a virus distantly related to Egypt Bee Virus was isolated in Japan.[12] This virus was temporarily termed the Japanese Isolate of Egypt Bee Virus, but was

quickly renamed for the characteristic symptoms it caused and given its present-day name: Deformed Wing Virus.[12]

DWV is the most widespread of all honeybee viruses, affecting 50-75% of colonies across the world.[10] This virus can act as a devastating long-term stressor to a hive at both the individual and colony levels, causing both CCD and increased winter mortality losses.[10,13] At the individual level, the classical symptoms of DWV infection in adults include, but are not limited to: shrunken and crippled wings; disfigured or discolored abdomens; protruded proboscis; and incomplete development of the hypopharyngeal glands and mandibular glands.[14] In larvae, pupae, and emerging pupae, infected individuals exhibit significantly higher mortality rates before capping, during pupation, and during emergence from capped brood cells; slower emergence from capped brood cells; and lower body weights.[14,15] Infections are not always overt; covert infections are the most common and occur when the viral load threshold to display visible morphological symptoms is not reached.[10] Covert infections have long manifested themselves in hives without causing extensive damage to colonies; severe overt infections are a mostly modern development. For an infection to be considered covert, it must meet three criteria: presence of viral material without symptoms of disease, spread via vertical transmission, and the occurrence of sporadic overt outbreaks.[12] Covert infections are also detrimental, leading to issues such as cognitive impairments, accelerated life cycle behaviors, increased rate of cellular apoptosis, and reduced life expectancy.[10,14] There are also disputed claims that covert DWV infection may lead to shortened flight duration and distance during foraging.[10] Individuals with visible overt

infections have a significantly higher viral load than their unaffected counterparts, containing a factor of approximately 1.7 x 10^6 more virus particles.[14]

At a colony level, individuals with both overt and covert DWV infections contribute to elevated hive mortality rates and ultimately CCD. Individuals with morphological symptoms of the wings, abdomen, and proboscis may have compromised flight, digestive, and feeding abilities – all of which contribute to early mortality. The implications of covert infections can also be highly deleterious.[10] These individuals often demonstrate behaviors earlier in their life cycle than what is natural, especially precocious foraging, or premature foraging, which is the natural response of bees under stressful conditions and is considered a risky behavior.[10] As these individuals live an abbreviated life, their total activity and contribution to the hive is also reduced.[10] Precocious foraging is also disruptive inside the hive, as bees demonstrating accelerated behavioral maturation spend less time in the nursing phase before beginning to forage, leaving a suboptimal number of individuals left tending to the brood and thus further accelerating colony collapse.[10] Both covert and overt infections can lead to neurological maladies including sensory defects, upregulation of the immune system, and memory deficits.[10]

Structure and Classification of Deformed Wing Virus

DWV belongs to the genus Iflavirus within the family Iflaviridae.[12] The virus consists of a single-stranded, positive-sense RNA genome (+ssRNA) that produces a non-enveloped virion 30 nm in diameter with icosahedral geometry and three major structural viral proteins: VP1, VP2, and VP3.[12,14,16] In general, all strains of DWV have

the following basic genome organization: a long 5' untranslated region (5' UTR) containing an Internal Ribosome Entry Site (IRES), a single open reading frame (ORF) in the middle, and a short 3' UTR that is highly conserved and terminates with a 3' poly-A tail.[12,17] It is believed that the IRES makes it possible for the virus to avoid and/or disrupt the host organism's cap-dependent mRNA translation system.[17]

Currently, there are two main strains of DWV described: Type A and Type B, which are believed to have diverged from each other around the year 1835.[2,18] There is also emerging evidence of a Type C variant that diverged around the year 1697.[18] These strains differ slightly in geographic distribution and Type B has been found to be more virulent.[2,10] Despite these slight regional variations, the DWV genome sequence is highly conserved and largely consistent throughout the world and among all strains.[4] DWV is closely related to Kakugo Virus (KV) and Varroa Destructor Virus 1 (VaDV-1), two other iflaviruses that affect honeybees.[4] Due to the 97% sequence homology between DWV and KV, there is ongoing research to determine whether these viruses are separate species or simply regional isolates of the same species.[4]

DWV Transmission and Infection

The primary mechanism of DWV transmission is via the parasitic *Varroa destructor* mite, a natural host of the virus.[13,14] This mite serves as both a biological vector that supports the replication of DWV before transmission and a mechanical vector that circumvents the *A. mellifera* physical defense barriers by injecting the virus directly through the exoskeleton and basal lamina of the epithelia and directly into the hemolymph.[1,19] While *Varroa* mites serve as the most common and infectious vehicle of

DWV transmission, once the virus is present in a colony it can also be spread by infected *A. mellifera* via horizontal transmission (intra-generational) and vertical transmission (inter-generational).[12] Vertical transmission of DWV occurs when infected parents produce infected offspring due to the presence of DWV in the sperm and/or egg gametes. Horizontal transmission of DWV occurs when the virus is spread between individuals from fecal matter, cannibalization, or orally via trophallaxis.[12,19] Active infection is best identified by detecting viral proteins and nucleic acid species produced during DWV replication via RT-PCR.[12,20]

The mechanism of entry into host cells for iflaviruses is believed to resemble that of the related family of picornaviruses, which utilize receptor-mediated endocytosis.[16] To enter endosomes, virions must be exposed to a solution of sufficient pH or ion concentration that initiates the detachment of the P domain at the C-terminal of VP3.[16] DWV's P domain contains a catalytic triad of the residues Asp294, His277, and Ser278.[16] These residues are structurally flexible, and it is hypothesized that when in the optimal conformation, this triad catalyzes hydrolytic reactions that allow the P domain to bind to virus receptors or disrupt the membrane in a fashion that makes it possible for the virus to inject its +ssRNA genome into the host cell's cytoplasm and infect the cell.[16]

Once an *A. mellifera* host is infected, DWV infection has a high degree of tissue specificity.[19] DWV RNA can be found in the brain, midgut epithelium, gut contents, and reproductive organs.[4,19] Viral loads are highest in the reproductive organs; the testis and seminal vesicles of drones and the ovaries of queens.[19] High viral loads in drones' reproductive organs can hinder their reproductive fitness and cause them to transmit DWV RNA through their sperm, thus contaminating the queen and contributing to the

vertical transmission of DWV to the next generation of workers.[19] DWV can also be found in multiple regions of the brain of infected *A. mellifera,* especially the corpora pedunculata neuropils, or mushroom bodies, where DWV actively replicates.[4,10] These structures serve as important higher brain centers that are largely responsible for olfaction and other sensory processes.[4] DWV infection of the mushroom bodies likely contributes to issues with olfaction, vision, and possibly even behaviors that promote the horizontal transmission of the virus to nearby colonies.[4,10] DWV has also been associated with memory loss, learning defects, and poor orientation abilities, making infected individuals more susceptible to predators.[10]

A. mellifera has limited innate immunity against DWV and largely ineffective defense mechanisms against *Varroa destructor* and the spread of the virus. Unlike *Apis cerana*, the eastern honeybee and original host of *Varroa*, *A. mellifera* cannot combat varroosis by temporarily suspending worker reproduction or entombing infested drone brood, processes which have shown to reduce the number of viable hosts for *Varroa* and reduce mite prevalence by up to 25% in *A. cerana*.[21,22] Instead, the *A. mellifera* mite defense strategy relies on grooming and hygienic activity.[21,23] Grooming is a behavior where bees inspect and clean their bodies to remove any foreign material, including mites. Bees may perform this task on themselves (auto-grooming) or on other individuals in the colony (allo-grooming).[24] Hygienic behavior describes the removal of dead, defective, diseased, or infested brood prior to emergence as adults.[13] Colonies that demonstrate high performance in grooming and hygienic behaviors represent the most attractive candidates for the selective breeding of mite-resistant honeybees.[13] There is also evidence that *A. mellifera* selectively bred for the trait “Suppression of Mite

Reproduction" (SMR; a variety of behaviors and detection mechanisms that result in heightened sensitivity to cues associated with mite-infested pupae) remove an even greater amount of mites than those bred for only hygienic activity.[25] SMR bees may also induce physiological changes in *Varroa* that reduces the mites' fertility and offspring viability.[25] This ability comes with a steep tradeoff, however, as this same factor is also believed to hinder pupal development and reduce overall brood viability.[25] Despite these attempted defensive measures, this host-parasite relationship is unbalanced and *A. mellifera* is still largely unequipped to effectively combat the invasive *Varroa* mite and ensuing DWV infection.[13]

Varroa Destructor Mites

Varroa destructor is a hematophagous ectoparasitic mite that poses a serious threat to modern apiculture.[13] This invasive species was spread to *A. mellifera* in Europe from its original host of *A. cerana* in Asia as domesticated honeybees were transported throughout the world during the 20th century, particularly the 1970's and 1980's.[12,13] Today, the only remaining *Apis* colonies free from *Varroa* exist in Australia and a few remote, isolated islands.[26] *Apis mellifera* is the only *Apis* species that does not naturally host a parasitic mite in its brood, making it especially susceptible to invasive mite species such as *Varroa.*[26] *Varroa* is best known for its association with the transmission of DWV to honeybee colonies via feeding behavior, the most destructive consequence of varroosis.

While DWV is occasionally found in colonies free of *Varroa,* the virus is detected in just 6-13% of colonies without the mites and in 75-100% of colonies infested by the

mites.[1,2] Viral load is also greatly amplified by the presence of these pests; there is a one-million-fold difference in viral load between colonies with *Varroa* compared to those without *Varroa*.[1,2] The seasonal levels and distribution of DWV also closely match the seasonal prevalence of *Varroa,* with levels climbing during the late summer.[12] While varroosis increases the viral load of DWV in a colony, it decreases the variant diversity.[2] It is believed that *Varroa* selects for the DWV strains with competitive advantages that cause them to be the most virulent and persistent in the local *A. mellifera* population.[2] It should also be noted that for a mite to cause an overt infection, it must be both a mechanical and biological vector of DWV that produces a high enough viral titer to reach the threshold of disease via transmission.[27] Mites that are capable of causing wing deformities contain 10^2 to 10^4 more DWV particles than mites that are not.[14]

When left untreated, most colonies will collapse from varroosis in 2-3 years.[13] Much like the associated DWV infection, *Varroa* infestation damages *A. mellifera* at both the individual and colony levels. *Varroa* is capable of injecting viral material and salivary proteins that activate latent infections present from vertical transmission directly into the hemolymph of its host.[28] It also consumes a significant amount of the host's hemolymph throughout all life stages.[13] This loss of hemolymph in adult honeybees weakens them and causes immunosuppression, but it is especially detrimental when it occurs during the larval and pupal stages.[13] When parasitized by *Varroa* during the developmental stages, worker and drone brood that survive the pupal phase will emerge with a reduced body weight and likely display symptoms of overt DWV infection.[15] The presence of just one adult female mite in a brood cell and her progeny that begin feeding during the pupal phase can reduce emergence weight of adult drones by 10%.[15] At the

colony level, *Varroa* inhibits colony reproduction by reducing the reproductive fitness of drones.[28] As less reproduction occurs and more workers die from DWV infections, the colony's overall foraging activity decreases, leading to a depleted nectar and pollen supply.[10] Additionally, colonies affected by varroosis exhibit less swarming.[29] Sometimes referred to as colony fission, swarming is the mechanism through which an entire colony reproduces by splitting one colony into two.[29]

The *Varroa* life cycle is divided into two major phases: phoretic and reproductive.[13] During the phoretic phase (sometimes referred to as the dispersal phase) mites attach to mature *A. mellifera* and travel with them as they forage, offering them a means of transportation to spread beyond the colony.[13] *Varroa* much prefers the dark, humid climate inside the hive – especially within sealed brood – so the dispersal that occurs during the phoretic phase marks the only time *Varroa* leaves the colony during its life cycle.[13] During the reproductive phase, *Varroa* focuses its efforts on a colony's brood.[13] This phase begins when mites enter worker and drone brood cells just prior to capping (t = 7-8 days after *A. mellifera* eggs are laid).[13] Mites preferentially choose cells that are shallower, wider, older (contain more residual semiochemical attractants from larval food and cocoons), and contain the largest larvae (preferably 5th instar).[30,31] Next, they crawl to the bottom of the cell where they "hide" beneath the larvae in the larval feed to avoid detection by hygienic bees until capping occurs.[13] After capping, mites feed on pupal hemolymph while undergoing oogenesis and vitellogenesis.[13] Mite eggs are laid about 70 hours after capping occurs and will develop into adult *Varroa* in approximately six days.[13] Finally, both parent and progeny emerge from the brood cell with the adult bee when pupation is completed (for workers, t = 20-21 days after *A.*

mellifera eggs are laid; for drones, t = 24 days).[13] Some may remain attached to the emergent bee while others will scatter throughout the colony. Of those that scatter, *Varroa* females can detect age and function of bees in the colony, and they methodically seek out middle-aged nurse bees to transport them to brood cells around the hive.[13]

During reproduction, *Varroa* are believed to prefer drone brood over worker brood due to drones' longer pupation period.[13] With the additional 3-4 days to feed and reproduce while pupae are capped, mites in drone cells will produce approximately 1.6 times more mature female offspring than their counterparts in worker cells.[13] *Varroa's* tendency to thrive in brood cells with longer pupation periods may also contribute to *A. mellifera's* elevated susceptibility to *Varroa* compared to *A. cerana,* a species with slightly shorter larval and pupal periods. Given this preference, it is estimated that drone brood have an 8-10-fold higher rate of infestation than worker brood.[30] This may also be due in part to the more intensive and frequent tending that drone brood receives, allowing more opportunities for mites on nurse bees to reach 5th instar drone larvae.[30] *Varroa* females are able to identify their preferred targets due to their attraction to methyl and ethyl esters of straight-chain fatty acids, brood pheromones present in the larval cuticle that stimulate capping of the brood cell.[32] These esters are inherently higher in drones and 5th instar larvae.[32] Mites are highly sensitive to changes in these pheromones; the variation in ester hydrocarbon structure between 4th and 5th instar larvae elicits a strong preference for 5th instar larvae.[31] Unlike worker and drone brood, queen brood show an exceptionally low rate of *Varroa* infestation.[33] This is likely explained by both the shorter pupation period of queen brood and the presence of octanoic acid in queen larvae and queen extracts, a compound known to be repellent to female *Varroa*.[13,33]

American Foulbrood and Paenibacillus Larvae

Paenibacillus larvae (P. larvae), formerly known as *Bacillus larvae*, is a Gram-positive and spore-forming bacterium that causes the disease American foulbrood (AFB) in *A. mellifera*.[34,35] The bacterium has a rod (bacillus) shape, rounded ends, a length of 2.5 – 5.0μm, a width of approximately 0.5μm, and often grows in elongated chains.[36] This facultative anaerobe produces highly infectious spores and it can be found in most parts of the world and in all castes within infected honeybee colonies.[34,37] Previously, *P. larvae* was separated into two subspecies, *Paenibacillus larvae larvae* and *Paenibacillus larvae pulvifaciens*.[38] However, a 2006 taxonomic review supported the merger of the two subspecies into one species based on biochemical profiles and spore morphologies.[38] There are currently four strains of *P. larvae* that have been identified to cause AFB, labelled ERIC I-IV based on their enterobacterial repetitive intergenic consensus (ERIC) sequences.[39] ERIC I is found worldwide, while ERIC II is found only in Europe and is considered the most virulent strain, as it is capable of killing all larvae in a hive within seven days of infection.[39] *P. larvae* produces the toxin C3larvin, which possibly contributes to AFB pathology.[39]

AFB affects a colony's brood and is characterized by disproportionately high mortality among larvae and pupae, while adults are not susceptible to the disease.[5] A larva must only consume a few spores within the first 36 hours of life post-hatching for infection to begin.[39] Given the severe pathology of the brood, AFB is currently the most damaging and economically relevant bacterial disease facing honeybees as it is ultimately lethal to the entire colony if left untreated.[5,34] Much like DWV, AFB can be transmitted

both horizontally, as bees travel throughout their colony, and vertically. Vertical transmission can occur at either the individual level when an infected queen or drone passes the virus to offspring during reproduction, or at the colony level when an infected colony swarms to "reproduce" or create a new colony. This disease is listed in the Terrestrial Animal Health Code through the World Organization for Animal Health, setting many strict recommendations on foreign honeybee importation to prevent further spread of this disease.[34,40]

P. larvae and AFB clinical infections can be easily identified in a hive by assessing the distribution and appearance of larvae in areas containing brood. Hives suffering from AFB will display empty or discolored spots within brood regions due to brood cells that are empty or contain dead or dying larvae.[35] Diseased hives also produce significantly less honey than their healthy counterparts.[41] Clinical symptoms of infected brood include discoloration, a characteristic foul odor, and brown remains with a gelatinlike consistency.[35,38] Freshly deceased, these remains are stringy and form rope-like strands when prodded.[38] During decay, these remains will shrivel into solid scales containing millions of active *P. larvae* spores that cling to the bottom and sides of brood cells.[38] Each infected larva may produce anywhere from 1 billion to 2.5 billion spores.[36,40] These scales are so firmly adhered to the brood cells that they are largely resistant to hygienic honeybee behavior, leaving them in place to release spores and ultimately infect the next larva that occupies the cell. Spores can remain active inside a hive for at least 35 years.[42] Subclinical infections must be diagnosed in a laboratory setting, where the presence of *P. larvae* is confirmed via microscopy and/or culture.[35,40]

Treatment of AFB is difficult given *P. larvae* spores' extreme heat and chemical resistances.[40] The pathogen has also developed resistance to many commonly administered antibiotics such as oxytetracycline (the most common AFB antibiotic of the last 50 years; commercially known as Terramycin and produced by Pfizer), which is the only antibiotic currently approved by the United States Food and Drug Administration for AFB treatment.[5,43] Oxytetracycline aims to thwart *P. larvae* spore germination until brood have passed the age of highest vulnerability while also buying the colony time to remove already dead or diseased brood.[43] While some antibiotics can effectively neutralize vegetative *P. larvae,* they still fail to treat spores and ultimately fail to prevent the recurrence of AFB.[35] To prevent the spread of the pathogen, it is therefore common practice to destroy diseased colonies, usually by burning them and the equipment associated with them.[5,35] Artificial swarming is also an option, a practice that involves prompting adult bees to relocate to a new hive and leave behind the diseased brood, which can then be destroyed.[44] Finally, colonies that are selectively bred for increased hygienic behavior in nurse bees have displayed a significant reduction in AFB prevalence and a much higher recovery rate.[41] Spores, the agents of AFB infection, begin appearing in diseased brood cells 10-11 days after eggs hatch when 5th instar larvae are developing into prepupae.[41] Sporulation occurs when the prepupae succumbs to the infection, so it is critical to the survival of the hive for hygienic bees to remove diseased larvae as quickly as possible.[45] Remarkably, data shows that *P. larvae* strains that are more virulent to individual larvae are less virulent to the overall colony, as the diseased larvae die faster and hygienic bees can remove them faster, leaving less time for bacterial propagation.[46]

Immunoglobulin Y

Immunoglobulin Y (IgY) is a newly utilized antibody with broad therapeutic capabilities to combat many pathogens.[47] This antibody was first described in 1983 and began to be applied for practical applications in the 1990's.[48] IgY can be found in the egg yolks of reptiles, amphibians, and most notably birds – specifically chickens.[48] This avian antibody is a result of passive immunity, as IgY is transferred from the maternal blood serum to egg yolks to protect developing embryos from the vertical transmission of pathogens throughout embryogenesis.[48,49] Maternal IgY also functions to provide protection to offspring early in life while the immune system matures and generates immune effectors.

IgY has a great deal of industrial appeal, as chickens can efficiently produce highly specific antibodies against a multitude of pathogens beyond just avian species.[50] Hens may be vaccinated with an antigen to produce IgY against the antigen of interest or with gene expression vectors to produce IgY with specificity for the given gene.[48] Evidence suggests that hens produce maximal IgY specific to the antigen or gene of interest when they are immunized intramuscularly in the pectoral muscle tissue as compared to receiving the immunization subcutaneously.[51,52] At peak immunity, 2-10% of hens' total IgY production will be specific to the antigen or gene of interest from the immunization.[53]

Some of the main advantages of IgY include its relative ease of mass production, the non-invasive manner in which it is collected, and its enhanced response to mammalian antigens.[50] Mature eggs contain 100-200 mg of IgY when they are laid, and one laying hen produces an average of 40 g of IgY per year.[48,50] There have been several

strategies developed to efficiently extract IgY suitable for biomedical use. Mammalian IgG and other types of passive antibodies have historically been utilized for such biomedical purposes, which required bleeding of hyperimmunized animals to harvest antibodies.[48] Using the IgY of chicken egg yolks represents a far more ethical, more cost-effective, and less invasive means of antibody extraction than the traditional practices of IgG collection.[48] There is also evidence that chickens can produce higher titer antibodies against mammalian antigens than other species vaccinated against these same antigens.[50] This is due to the increased phylogenetic distance between chickens and mammals, as immune response increases as the evolutionary gap increases between antigen host species and immunized species.[50,54]

IgY has the classic "Y" antibody shape and consists of the standard two identical heavy chains and two identical light chains that are connected by disulfide bonds.[55] These chains consist of constant domains and variable domains that make up antigen-binding fragments (FABs) that bind specific antigen sequences. Light chains are comprised of one variable domain and one constant domain. Heavy chains consist of one variable domain and four constant domains (C_H1, C_H2, C_H3, C_H4).[54] Perhaps the most prominent and unique structural feature of IgY is its lack of a hinge region, making it more rigid than mammalian immunoglobulins, including IgG.[54] This lack of flexibility is caused by glycine and proline residues between the C_H1, C_H2, and C_H3 constant domains.[55] It is believed this extra rigidity contributes to the functionality of IgY. This includes IgY's inability to agglutinate polyvalent antigens in normal conditions, likely as a result of the inability to achieve the required conformation of the FABs due to steric hindrance.[55] IgY has a molecular weight of approximately 180 kDa.[55]

CHAPTER TWO

Materials & Methods

Vaccination of Chickens and Yolk Collection

Six white leghorn chickens, approximately 6 months of age, were immunized four times with 10 µg each of purified DWV VP1, VP2, and VP3 proteins in phosphate buffered saline along with incomplete Freund's adjuvant at 67% (v/v). The DWV proteins were expressed as hexahistidine-tagged fusion proteins in baculovirus and purified using affinity chromatography by Genscript. An additional six birds of the same age were also immunized four times against *P. larvae.* These vaccinations consisted of spores cultured from *P. larvae* obtained from the American Type Collection Collection and grown on 5% sheep blood agar for seven days. Spores were recovered by scraping the plate overlaid with 5 mL of PBS. Spores were inactivated with formalin.

The birds were vaccinated with a 0.5 cc intramuscular injection in each breast. Immunizations were given periodically at 21-day intervals to sustain the immune response and trigger the immunized hens to continue passively generating pathogen-specific antibodies in their eggs. Eggs were collected daily, beginning two weeks after the third vaccination. Yolks were separated from the albumen using a wire egg separating apparatus. The yolks were pooled and refrigerated until further processing.

Figure 2-1. Separated egg yolks from hens vaccinated against DWV and *P. larvae*.

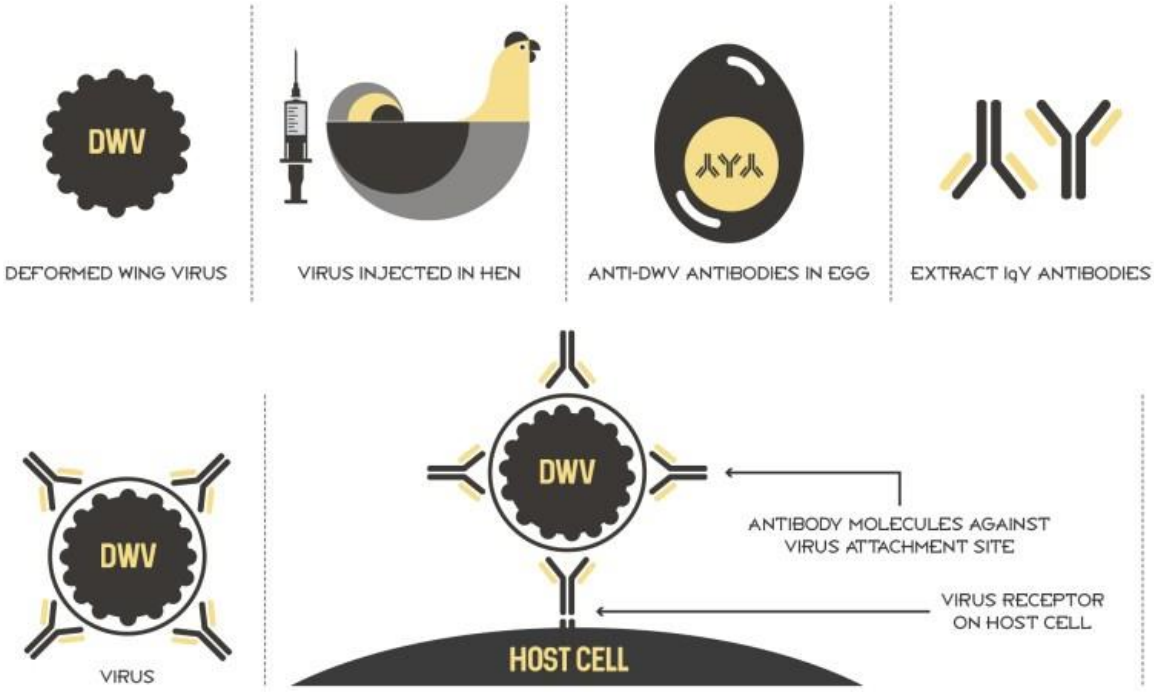

Figure 2-2. Visualization of IgY experimental concept.[56] Retrieved from https://tallgrassbiologics.com/services/

Purification of Antigen-Specific IgY via Freeze-Thaw

Yolks were processed according to Stage I of the protocol described by Petr Hodek.[50] Yolks were diluted with seven volumes of tap water and brought to a pH of 5.0 using 1.0 N HCl. This was done in 1-gallon containers, with each container consisting of 500 mL yolk, 3 L water, and 36 mL 1.0 N HCl. A pH probe was used to verify the pH of each aliquot after mixing. This solution was frozen overnight at -18°C and thawed the following day at room temperature.

Upon thawing, the solution separated into two distinct layers: an upper aqueous layer containing the IgY (mostly transparent) and a lower viscous layer, containing lipids and other waste (orange). The upper aqueous layer was removed with a pipette connected to a transfer pump with the bottom lipid layer being discarded. The aqueous layer was labelled and referred to as "freeze-thaw" or "Diluted and Clarified Egg Yolks". The Diluted and Clarified Egg Yolks were then poured through cheese cloth and a mesh wire filter to remove any remaining large lipid particles.

Figure 2-3. Diluted and Clarified Egg Yolks after lipid layer is discarded. Note the off-yellow, cloudy color.

Next, the Diluted and Clarified Egg Yolks were concentrated with the MiniKros Hollow Fiber Filter Module (P/N: N04-E030-05-N; Media/Rating: mPES / 30 k; Surface Area: 5400 cm^2; Max.Op.Pressure 30 psig (2 bar); SN: 3303990-07/18-002) using the Masterflex Industrial/Process Easy-Load™ Pump Head (model 7529-30). The pump was calibrated to a flow rate of 700 mL/minute.

Figure 2-4. Filtering apparatus used to concentrate Diluted and Clarified Egg Yolks into Hollow Fiber Concentrated Antibody.

The filter concentrated the antibody solution until only foam remained in the line, and this concentrate was added to an equal volume of PBS and stored at -80°C. This final 1:1 solution of concentrate and PBS was labelled and referred to as "Hollow Fiber Concentrated Antibody". The filtrate from the permeate line was also saved and labelled as "Hollow Fiber Filtrate". It should be noted that this highly concentrated antibody

separates into two distinct layers when refrigerated and should be shaken vigorously to homogenize before use.

Figure 2-5. Hollow Fiber Concentrated DWV and *P. larvae* IgY and corresponding filtrate, respectively.

Purification of Antigen-Specific IgY via Exalpha EggsPress Kit

For use as a standard with which to compare the Hollow Fiber Concentrated Antibody, a small aliquot of yolk was also processed via the EggsPress IgY Purification Kit from Exalpha. This kit offers an efficient way to purify IgY from the egg yolks of immunized hens for research purposes without the loss of antibody activity.[57] 100 mL of yolks were added to a 1 L jar along with 5 volumes of cold Reagent A and gently stirred.[57] This solution was incubated for two hours in the refrigerator. After incubation,

the solution was shaken for homogenization and then centrifuged at 4,000 x g for 20 minutes at 4°C. The resulting colorless supernatant (400 mL) was transferred into a graduated cylinder and mixed slowly with an equal volume of Reagent B, stirring for two minutes.[57] This suspension was then incubated a second time in the refrigerator for two hours. After incubation, the suspension was shaken to homogenize and centrifuged at 4,000 x g for 20 minutes at 4°C. After this centrifugation, the supernatant was discarded. 10 mL PBS was added to the first centrifuge tube, pipetted up and down to dissolve the pellet, and then transferred to the successive centrifuge tube, a process that was repeated until the pellets from all 10 centrifuge tubes were pooled together and suspended in the same 10 mL of PBS. This entire process was performed on 100 mL of yolks from both DWV- and *P. larvae*-vaccinated hens.

IgY ELISA

An indirect enzyme-linked immunosorbent assay (ELISA) was performed to quantify the amount and activity of antigen-specific IgY in each preparation. Immulon 2HB polystyrene microplates were coated with recombinant VP1, VP2, and VP3 by incubating plates at 4°C overnight with 100 uL per well of a 50 mM carbonate buffer, pH 9.6, containing 1 ug/mL of each protein.

To begin the assays, plates were emptied and 100 μL of Thermo Fisher Scientific's SuperBlock Blocking Buffer was added to each well using a multichannel pipette. The plates were incubated for one hour at 37°C. Following this first incubation period, the plates were emptied again and another 100 μL of SuperBlock was added to each well. Next, 100 μL of primary antibody diluted 1:50 in SuperBlock was added to row A, pipetted up and down for mixing, and transferred likewise through rows B-H to

give a series of dilutions. 100 µL was removed and discarded from row H, giving all wells a final total volume of 100 µL. The plates were then incubated a second time for one hour at 37°C. A table of the ELISA plate setup showing primary antibodies used and dilutions is shown below.

Table 2-1. Plate setup and dilutions for IgY ELISA.

	1	2	3	4	5	6	7	8	9
A	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	Blank
B	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	Blank
C	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	Blank
D	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	Blank
E	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	Blank
F	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	Blank
G	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	Blank
H	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	Blank

Primary antibodies for each column are as follows: 1 – DWV EggsPress Kit Aby; 2 – DWV Hollow Fiber Concentrated Aby; 3 – DWV Hollow Fiber Filtrate; 4 – DWV Diluted and Clarified Egg Yolks; 5 – *P. larvae* EggsPress Kit Aby; 6 – *P. larvae* Hollow Fiber Concentrated Aby; 7 – *P. larvae* Hollow Fiber Filtrate; 8 – *P. larvae* Diluted and Clarified Egg Yolks; 9 – SuperBlock Control (Blank)

Following incubation, plates were emptied and washed three times with 150 µL of a solution of phosphate buffered saline containing 0.05% tween 20 (PBS-tween) per well. Anti-chicken IgY horseradish peroxidase (HRP) served as the secondary antibody. This antibody was diluted 1:1000 in SuperBlock and added at a volume of 100 µL per well. The plates were then incubated a third time, this time for 30 minutes at 37°C. After incubation, plates were once again emptied and washed three times with 150 µL of PBS-

tween per well. Immediately following the final wash, 100 µL of SIGMA*FAST*™ OPD (*o*-phenylenediamine dihydrochloride), an HRP substrate, was added to each well and incubated for 15 minutes at room temperature. After 15 minutes, 100 µL of 1.0 N HCl was added to each well to halt the reaction. The optical densities of each well were then read at a wavelength of 460 nm using a Biotek plate reader.

Preparation of P. Larvae Challenge

Paenibacillus larvae was purchased from the American Type Collection Collection (ATCC 49843). *P. larvae* was propagated on tryptic soy agar containing 5% sheep blood at 37°C. Spores were isolated from 5-day cultures by flooding the plates with 3 mL of ice-cold PBS followed by gentle scraping with a sterile inoculation loop. Fluids were pipetted to a centrifuge tube and centrifuged at 1,000 x g for 5 minutes to pellet vegetative cells. The supernatant containing spores was transferred to a new tube.

Preparation of DWV Challenge

A lot of 320 frozen honeybees was purchased from a commercial supplier. Bees were frozen at -80°C followed by homogenization in a plastic liner containing 80 mL of PBS (10 mL PBS for every 40 bees) for a final volume of 88 mL. This solution was centrifuged at 5,000 x g for 10 minutes at 4-8°C. The insoluble orange portion of the supernatant and pellet were discarded, while the remaining supernatant was spun again at 20,000 x g for 20 minutes at 4-8°C. Again, the floating orange layer and pellet were discarded. The remaining supernatant was then centrifuged at 75,600 x g (25,000 rpm) for two hours at 4-8°C. The resulting pellet was suspended in 2 mL PBS, while the

supernatant was discarded. The temperature during all centrifugation steps should be held between 4-8°C. The challenge was stored at -80°C until use in larval challenges.

Larval Experiments

To test the effectiveness of the antibody, worker bee larvae were grown *in-vitro* according to the protocol described by Daniel Schmehl.[58] A frame containing at least 25% eggs and 1st instar larvae was selected from a hive and transported to the lab in an insulated box. The frame was kept humidified (i.e. draped in wet paper towels) during transport. Using a Chinese grafting tool, 1st instar larvae were grafted from the brood cells of the frame into queen cell cups (Mann Lake Ltd. Cat. #QC-520) inserted into each well of 48-well tissue culture plates. Each queen cup contained 20 μL of Diet A at the time of grafting. Larvae were fed according to Schmehl's feeding schedule (see Tables 2-2 and 2-3) with two exceptions: 20 μL of Diet A being administered on day 2 rather than 20 μL of Diet B; and an additional 10 μL of Diet A containing treatment was given on day 0, post-grafting.[58] Larvae receiving challenge received challenge only on day 0, while larvae receiving antibody received antibody at the desired concentration in each feeding. Rather than applying feed directly on top of larvae, it was pipetted down the side of the queen cups to avoid drowning.

Table 2-2. *Percent Composition of Larval Feed*

	Royal Jelly	**Glucose**	**Fructose**	**Yeast Extract**	**Water**
Diet A	44.25%	5.30%	5.30%	0.90%	44.25%
Diet C	50.00%	9.00%	9.00%	2.00%	30.00%

Antibody and challenge were added at desired concentrations.

Table 2-3. Larval Feeding Schedule

Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
20 μL Diet A 10 μL Diet A + Treatment	None	20 μL Diet A	30 μL Diet C	40 μL Diet C	50 μL Diet C

Days correspond to time since grafting. Antibody groups were fed antibody in each feeding, while challenge groups were only fed challenge on day 0.

Grafted larvae were then placed in a plastic container containing a supersaturated salt solution consisting of 80 g K_2SO_4, 200 g NaCl, and 500 mL H_2O and incubated at 35°C in a humidified incubator. For both the DWV and *P. larvae* models, there was a negative control group receiving only feed and three Hollow Fiber Concentrated Antibody control groups without challenge (1:25 dilution, 1:100 dilution, and 1:400 dilution) to test for toxicity. Both models also had a positive control group receiving either DWV stock diluted 1:10 in larval feed (Type A DWV at 10^7 genome copies per

larvae) or *P. larvae* spore challenge material diluted 1:10 in larval feed. Additionally, each model also had three final groups that received a 1:10 dilution of challenge material and one of the three aforementioned dilution levels of pathogen-specific Hollow Fiber Concentrated Antibody.

Mortality assessment and feeding occurred every 24 hours post-grafting. Dead larvae were identified by a combination of discoloration, a sunken appearance beneath the feed, and grossly apparent stunted growth relative to healthy control group individuals. All dead larvae were removed daily, sorted by treatment group, and frozen for molecular analysis. Larvae from each treatment group were separated into "live" and "dead" pools. Larvae were considered "live" only if they survived until the experiment's endpoint.

Note: It is critical to use *only* 1st instar larvae for both DWV and *P. larvae* challenges, as larvae are most susceptible to these pathogens during the first 24-48 hours of life, post-hatching.

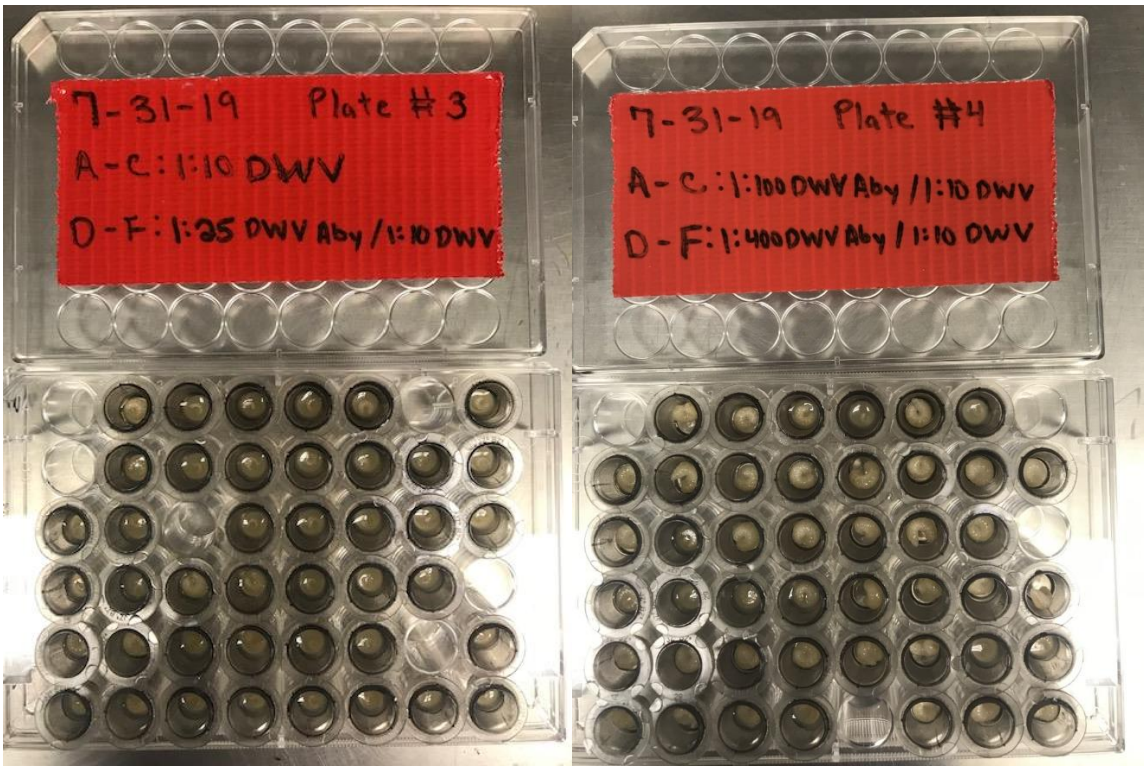

Figure 2-6. *In-vitro A. mellifera* larvae in queen cups during DWV challenge experiment. Dead larvae have been removed and pooled for further analysis.

DWV Real-Time PCR

At the conclusion of each DWV *in-vitro* larval experiment, larvae from each treatment group were tested for the presence of DWV using QuantiTect SYBR Green Real-Time RT-PCR Kit. Each pool of larvae was added to two volumes of PBS, crushed using a pipette tip, and vortexed for several seconds. 0.2 mL of each sample was added to a micro-centrifuge tube and spun at 2,000 x g for 5 minutes.

100 µL of each resulting supernatant was added to 100 µL of Thermo Fisher Scientific's PrepMan^{TM} Ultra and vortexed. These tubes were placed on a heat block at 98°C for 10 minutes. After heating, samples were micro-centrifuged at 20,627 x g

(15,000 rpm) for 10 minutes. The resulting supernatants were used as the template RNA samples for PCR. The following volumes (given per sample) were combined to create the master mix: 0.5 μL PAN DWV Forward primer, 0.5 μL PAN DWV Reverse primer, 12.5 μL 2X QuantiTect SYBR Green RT-PCR Master Mix, 8.75 μL nuclease-free water, 0.25 μL QuantiTect RT Mix, and 2.5 μL template RNA sample (total volume/well = 25 μL). Thermocycler settings are given in Table 2-4.

Table 2-4. Thermocycler Protocol Used for SYBR Green RT-PCR of DWV

Cycle	**Repeats**	**Step**	**Dwell Time**	**Set Point (°C)**
1	1	1	30:00	50.0
2	1	1	15:00	95.0
3	44	1	00:15	94.0
		2	00:30	55.0
		3	00:30	72.0

P. Larvae Plating

At the conclusion of each *P. larvae in-vitro* larval experiment, larvae were sorted by treatment groups and further separated into "live" and "dead" pools. Each set of larvae was pooled in a tube and centrifuged at 1,000 x g for one minute to bring the minimal volume of larval remains to the bottom of the tube. The remains were then suspended in two volumes of PBS and crushed using a pipette tip. The tubes were spun

again at 1,000 x g for one minute to remove large and insoluble debris. Using the resulting supernatant, a series of four dilutions in PBS was prepared for each sample: 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}. 100 μL of each dilution for each treatment was added to a tryptic soy broth + 5% sheep blood agar plate (BAP) and incubated at 37°C. Colony counts were taken on days two and five.

CHAPTER THREE

Results

IgY Concentration

Concentrated IgY solutions with anti-DWV and anti-*P. larvae* specificity were successfully created by processing the egg yolks of white leghorn chickens vaccinated against each respective antigen. It was estimated that each egg provides approximately 16.67 mL of yolk and 11.05 mL of Hollow Fiber Concentrated Antibody. Each 100 mL batch of egg yolks purified using the Exalpha EggsPress Kit yielded a final volume of 17 mL: 10 mL PBS and 7 mL of suspended antibody pellets.

The antibody titer was qualitatively determined by utilizing indirect enzyme-linked immunosorbent assay (ELISA), with VP1, VP2, and VP3 acting as the antigens for the anti-DWV ELISA and *P. larvae* as the antigen for the anti-*P. larvae* ELISA. For each model, Diluted and Clarified Egg Yolks from hens non-immunized for the antigen of interest were run as a control. The antibody titers for the various antibody preparations are illustrated below with absorbance readings charted as a function of dilution.

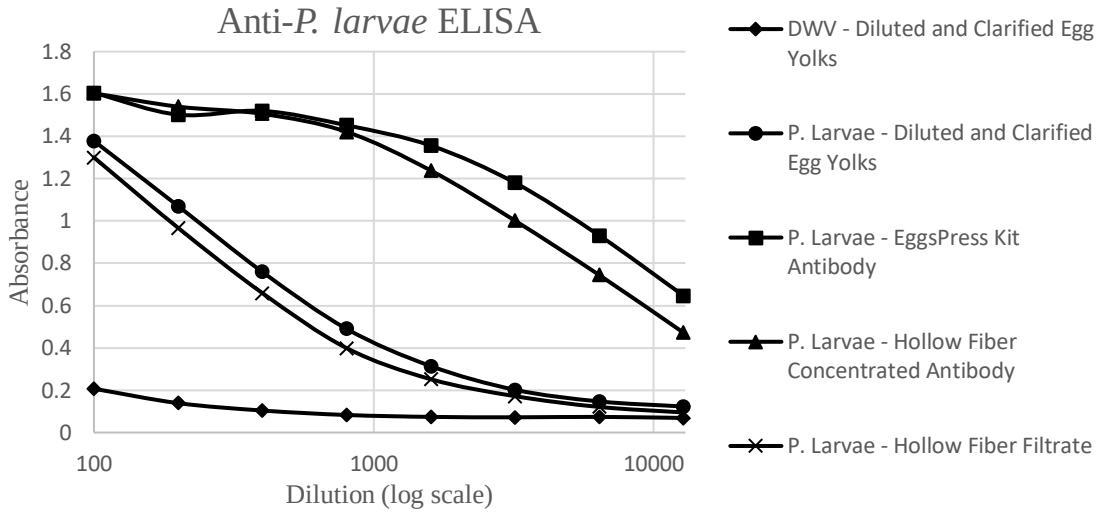

Figure 3-1. Enzyme-linked immunosorbent assay data for processed *P. larvae*-specific antibody. 1:250 dilution was used for primary antibody (various preparations of IgY) and 1:5000 dilution was used for secondary antibody (horseradish peroxidase). *P. larvae* was the antigen. Anti-DWV Diluted and Clarified Egg Yolks (non-immunized for *P. larvae*) were run as a control. "Kit Purified" refers to antibody concentrated using the Exalpha EggsPress IgY Purification Kit, which was used as a standard. "Filtrate" refers to the waste from the permeate line during filtration.

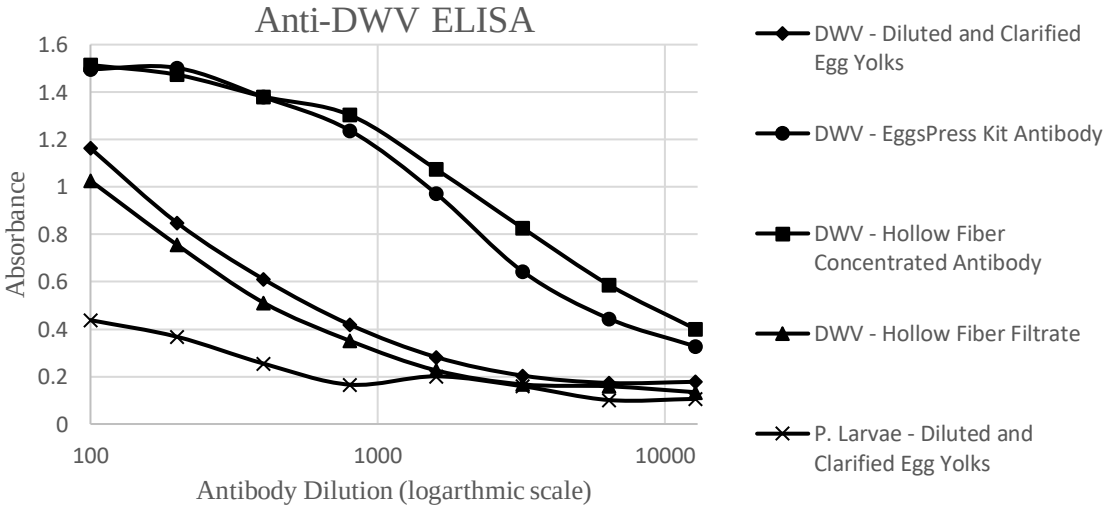

Figure 3-2. Enzyme-linked immunosorbent assay data for processed DWV-specific antibody. 1:50 dilution was used for primary antibody (various preparations of IgY) and 1:1000 dilution was used for secondary antibody (horseradish peroxidase). VP1, VP2, and VP3 proteins acted as the antigen. *P. larvae* samples were run as controls. "Kit Purified" refers to antibody concentrated using the Exalpha EggsPress IgY Purification Kit, which was used as a standard. "Filtrate" refers to the waste from the permeate line during filtration.

In both the anti-*P. larvae* and anti-DWV models, Hollow Fiber Concentrated Antibody demonstrated a much higher antibody titer than its unfiltered counterpart, the Diluted and Clarified Egg Yolks. The Hollow Fiber Concentrated Antibody also demonstrated a much higher antibody titer than the Hollow Fiber Filtrate, providing evidence that the filtering process was effective and that it efficiently captured the IgY. Additionally, the Hollow Fiber Concentrated Antibody either surpassed (in the case of the anti-DWV model) or nearly attained (in the case of the anti-*P. larvae* model) the standard of the Exalpha EggsPress IgY Purification Kit. In both models, the non-specific Diluted and Clarified Egg Yolks functioning as a control showed very low titers of antibodies that demonstrated activity against the pathogen of interest.

P. Larvae Challenge Model Larval Mortality

The *P. larvae* larval challenge model was carried out for three days. All antibody administered in this model was Hollow Fiber Concentrated Antibody. The daily mortality data is displayed in the following table.

Note: The *P. larvae* model used the antibody-only controls from the DWV model, as all component concentrations and preparation procedures of the solutions were entirely identical.

Table 3-1. *P. larvae* challenge model daily mortality (number of new larval deaths per day)

Treatment Group	Day 1	Day 2	Day 3	Total
Control (Feed Only)	0	1	0	1
1:25 DWV Aby	0	0	0	0
1:100 DWV Aby	0	0	0	0
1:400 DWV Aby	0	1	0	1
1:10 P.L. Spores	0	4	20	24
1:25 P.L. Aby / 1:10 P.L. Spores	0	2	16	18
1:100 P.L. Aby / 1:10 P.L. Spores	0	1	3	4
1:400 P.L. Aby / 1:10 P.L. Spores	0	2	12	14

These results revealed that among larvae receiving *P. larvae* spore challenge, those also receiving a 1:100 dilution of *P. larvae* aby (1:100 *P. larvae* Aby / 1:10 *P. larvae* spores) displayed by far the lowest mortality rates. Therefore, the 1:100 *P. larvae* Aby dilution was considered the minimum protective dosage to significantly reduce *P. larvae*-related mortality. Only the following four treatment groups were considered for statistical analysis: Control (Feed Only), 1:100 DWV aby, 1:10 *P. larvae* spores, and 1:100 *P. larvae* aby / 1:10 *P. larvae* spores. The cumulative mortality percentages of these four groups throughout the three-day duration of this experiment is shown in Figure 3-3.

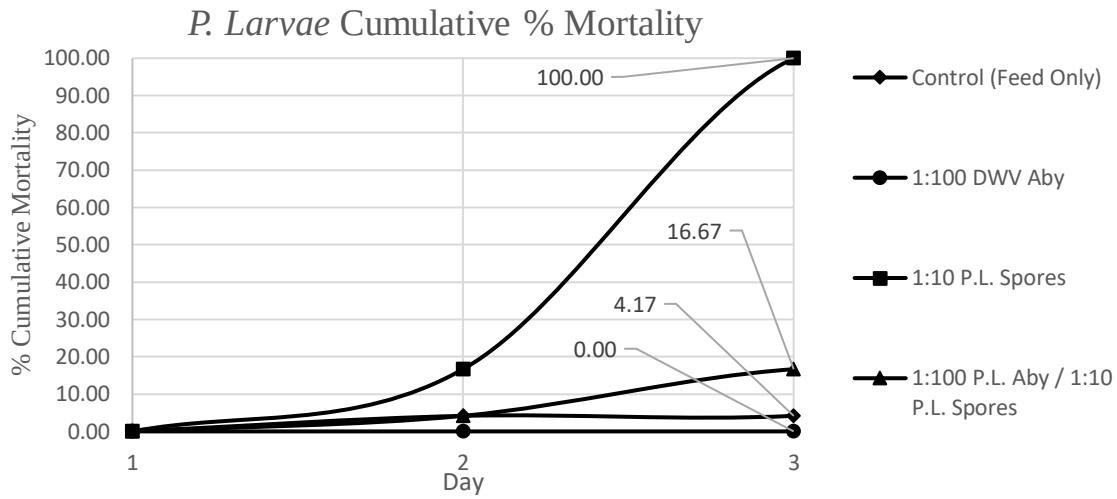

Figure 3-3. Cumulative larval mortality percentages through three-day *P. larvae* challenge model. Data labels display total group mortality percentages at the experiment's endpoint (t = 3 days).

The data was analyzed using the Kaplan-Meier method for survival analysis.[59] Using this model, it was assumed that the probability of individuals being censored was the same for all groups and did not affect the outcome, survival probabilities were the same for all individuals in all groups, and the event of interest (death) occurred at the specified times.[59] The Kaplan-Meier survival curves with 95% confidence intervals for each group are displayed below. Larvae surviving beyond day 3 are considered right-censored and are therefore indicated by a tick mark.

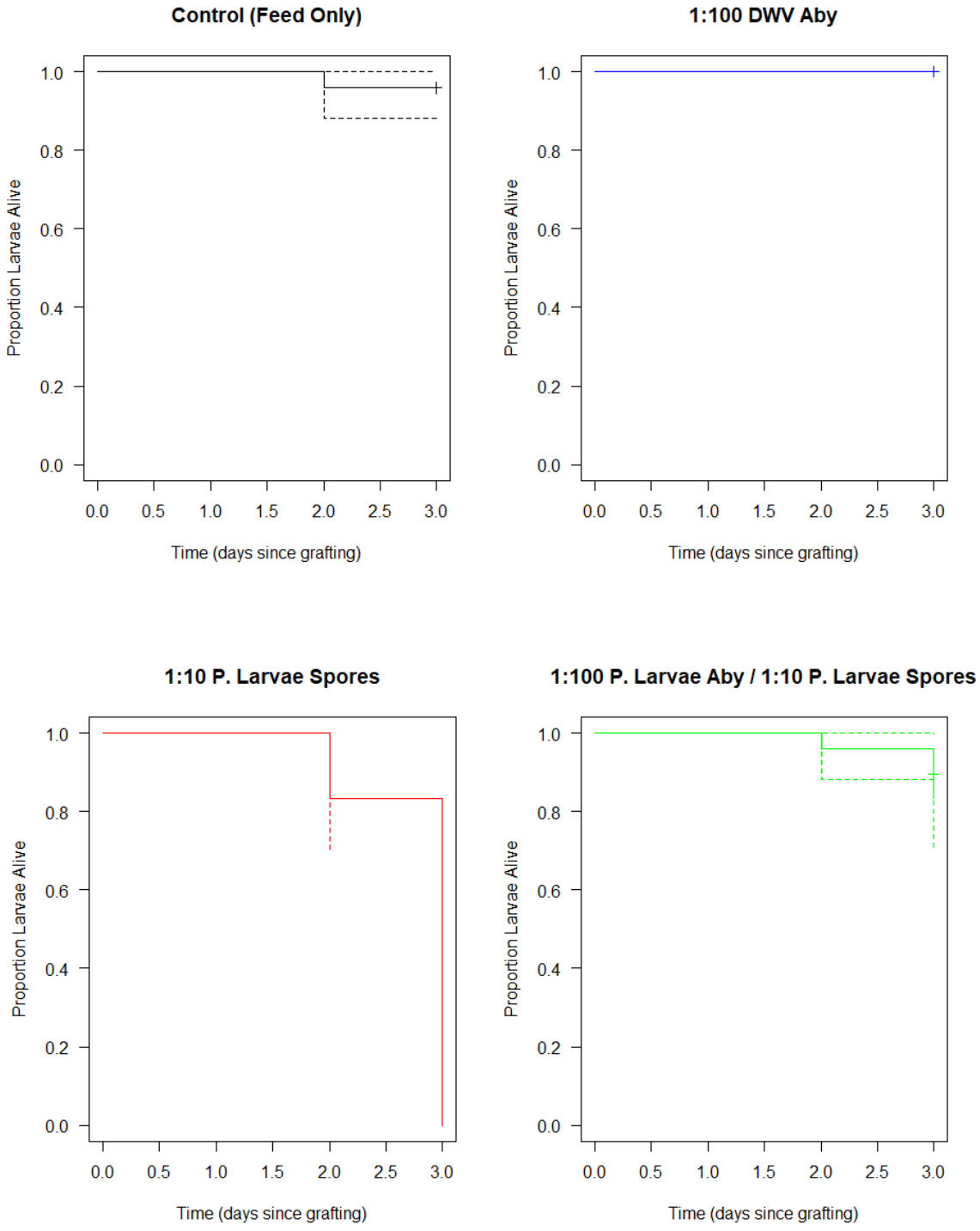

Figure 3-4. Survival curves for *P. larvae* model with 95% confidence intervals. Tick marks represent censored individuals (larvae that survived to endpoint).

The survival data of the four groups was then compared using the log-rank test, which operates under the same assumptions as the Kaplan-Meier method. The null hypothesis (H_0) for each comparison was that the mortality rates of the two groups being compared had no significant difference over the three-day experiment. The alternative hypothesis (H_1) was that one of the groups had a significantly higher mortality rate than the other over the three-day experiment. One degree of freedom (df=1) and a confidence level of 95% was utilized ($\alpha = 0.05$), meaning a p-value of less than 0.05 was sufficient to reject the null and imply a significant difference in mortality.

Four separate comparisons of mortality rates were made between groups. The first compared the control (feed only) group and the 1:100 DWV aby group to test for potential antibody toxicity. Log-rank analysis gave a p-value of 0.3, failing to reject the null and showing no significant difference between mortality rates. No difference in these mortality rates suggests that the 1:100 dilution of antibody was nontoxic.

The next comparison was between the control (feed only) group and the 1:10 *P. larvae* spore challenge group. This comparison functioned to test for pathogenicity of the *P. larvae* challenge material, and it yielded a p-value of 6e-10. This result rejected the null and implied a significantly higher mortality in the group receiving *P. larvae* spore challenge than in the control group receiving feed only. It was therefore assumed that the *P. larvae* spore challenge material was responsible for inducing a higher rate of mortality than was seen in the control group.

The third comparison was between the 1:10 *P. larvae* spore challenge group and the 1:100 *P. larvae* aby / 1:10 *P. larvae* spore treatment group. This comparison tested to see if larvae receiving antibody in addition to challenge material survived at a significantly

higher rate than larvae receiving only challenge material. This log-rank comparison returned a p-value of 4e-8, rejecting the null and suggesting that larvae receiving a 1:100 dilution of *P. larvae*-specific antibody in their feed in addition to the DWV challenge material had a significantly lower mortality rate and higher rate of survival than larvae receiving only challenge.

The last comparison was between the control (feed only) group and the 1:100 *P. larvae* aby / 1:10 *P. larvae* spore treatment group. This comparison tested for a potential difference in the mortality rate of larvae challenged with *P. larvae* spores and treated with antibody compared to larvae receiving no challenge or antibody. This log-rank analysis returned a p-value of 0.2, failing to reject the null hypothesis and implying that the larvae receiving both challenge and antibody treatment did not die at a higher rate than larvae receiving only feed in the control group.

P. Larvae Challenge Model CFU Evaluation

Colony counts from the dilution series plated with the prepared larvae from the *P. larvae* challenge model were taken on days 2 and 5 to calculate CFU/mL. The results are summarized in Figure 3-5.

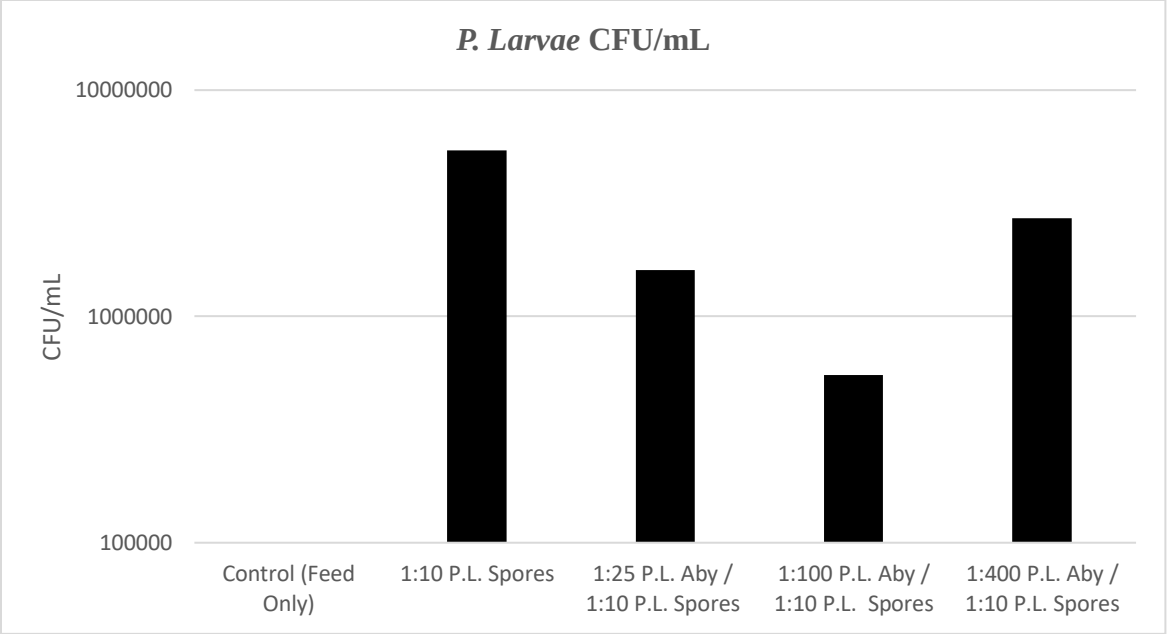

Figure 3-5. CFU/mL values of larvae from *P. larvae* challenge model.

The CFU data closely matched the mortality data; the feed-only controls were negative for *P. larvae* cells and the challenge group had the most viable *P. larvae* cells. More significantly, however, of the treatment groups receiving both challenge and anti-*P. larvae* IgY, the group receiving the 1:100 dilution of antibody demonstrated the fewest CFU/mL. The 1:100 *P. larvae* Aby / 1:10 *P. larvae* spores treatment group had both the lowest mortality rate and lowest CFU/mL value. This alignment with the mortality data reinforces the conclusion that the 1:100 dilution of anti-*P. larvae* IgY corresponds to the minimum protective dosage.

DWV Challenge Model Larval Mortality

The DWV larval challenge model was carried out for five days. All antibody administered in this model was Hollow Fiber Concentrated Antibody. The daily mortality data is displayed in the following table.

Table 3-2. DWV challenge model daily mortality (number of new larval deaths per group per day)

Treatment Group (n=24)	Day 1	Day 2	Day 3	Day 4	Day 5	Total
Control (Feed Only)	0	1	0	2	3	6
1:25 DWV Aby	0	0	0	0	0	0
1:100 DWV Aby	0	0	0	0	0	0
1:400 DWV Aby	0	1	0	0	0	1
1:10 DWV	0	1	1	2	15	19
1:25 DWV Aby / 1:10 DWV	0	0	2	0	8	10
1:100 DWV Aby / 1:10 DWV	0	1	0	2	4	7
1:400 DWV Aby / 1:10 DWV	0	0	0	1	5	6

This data demonstrates that among larvae receiving DWV challenge, those also receiving a 1:100 dilution of DWV aby (1:100 DWV Aby / 1:10 DWV) displayed among the lowest mortality rates. Therefore, the 1:100 DWV Aby dilution was considered the minimum protective dosage to significantly reduce DWV-related mortality. The following four treatment groups were considered for statistical analysis: Control (Feed Only), 1:100 DWV Aby, 1:10 DWV, and 1:100 DWV Aby / 1:10 DWV. The cumulative mortality percentages of these four groups throughout the duration of the experiment is visualized in Figure 3-6.

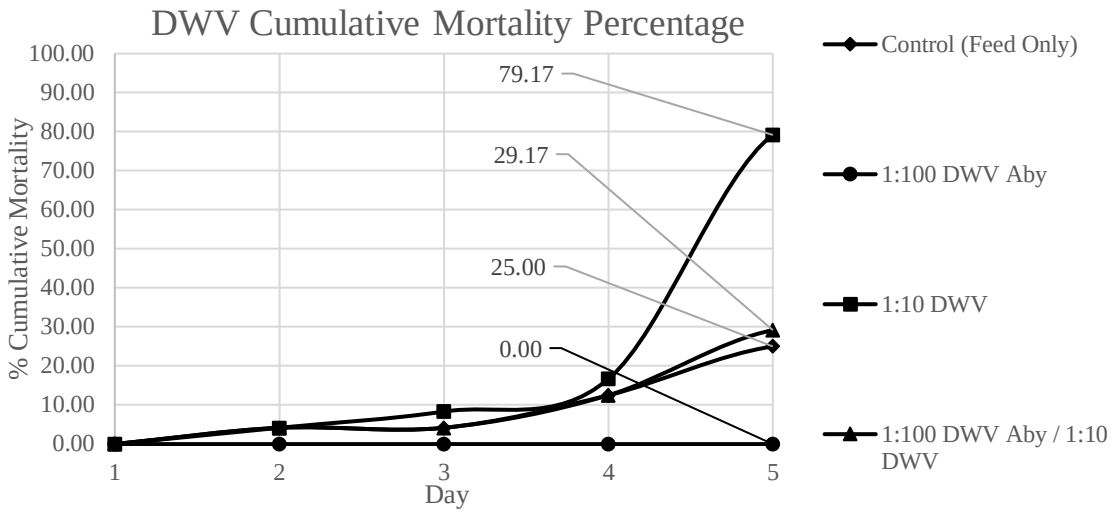

Figure 3-6. Cumulative larval mortality percentages through five-day DWV challenge model. Data labels display total group mortality percentages at the experiment's endpoint (t = 5 days).

The data was then analyzed using the Kaplan-Meier method for survival analysis.[59] Using this model, it was assumed that the probability of individuals being censored was the same for all groups and did not affect the outcome, survival probabilities were the same for all individuals in all groups, and the event of interest (death) occurred at the specified times.[59] The Kaplan-Meier survival curves with 95% confidence intervals for each group are displayed below. Larvae surviving beyond day 5 are considered right-censored and are indicated by a tick mark.

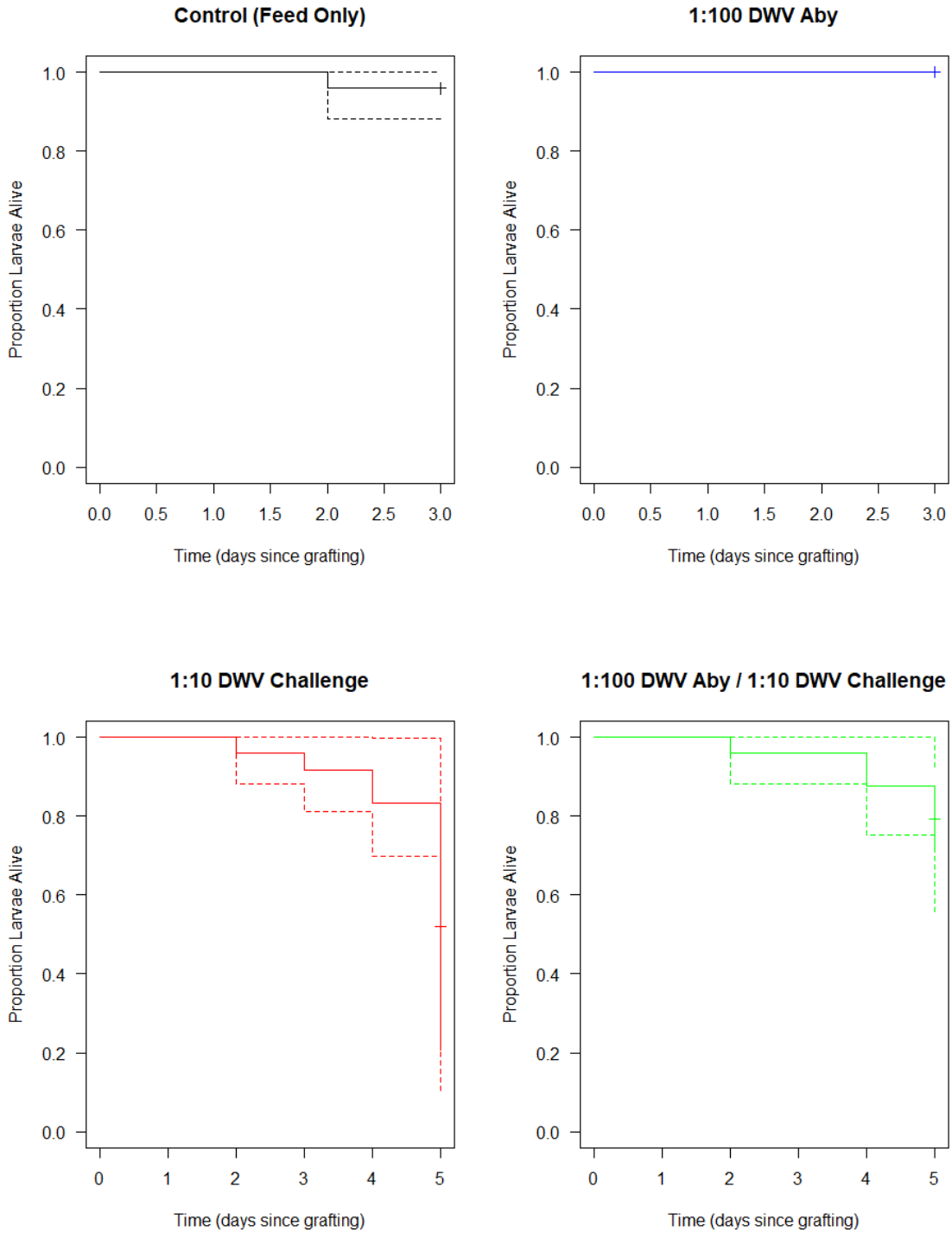

Figure 3-7. Survival curves for DWV model with 95% confidence intervals. Tick marks represent censored individuals (larvae that survived to endpoint).

The survival data of the four groups was then compared using the log-rank test, which operates under the same assumptions as the Kaplan-Meier method. The null hypothesis (H_0) for each comparison was that the mortality rates of the two groups being compared had no significant difference in mortality rate over the five-day experiment. The alternative hypothesis (H_1) was that one of the groups had a significantly higher mortality rate than the other over the five-day experiment. One degree of freedom (df=1) and a confidence level of 95% was utilized ($\alpha = 0.05$), meaning a p-value of less than 0.05 was sufficient to reject the null and imply a significant difference in mortality.

Four mortality comparisons were made between groups. The first comparison was between the control (feed only) group and the 1:100 DWV aby group to test for antibody toxicity. Log-rank analysis gave a p-value of 0.01, implying that the group receiving antibody had a significantly lower mortality than the feed-only controls. However, as zero larvae died in the 1:100 DWV aby group, it was assumed that there was no antibody toxicity.

The next comparison was between the control (feed only) group and the 1:10 DWV challenge group. This comparison was made to test for pathogenicity of the DWV challenge material and it yielded a p-value of 0.001. This result was sufficient to reject the null and implied a significantly higher mortality in the group receiving DWV challenge than in the control group receiving feed only. It was therefore assumed that the DWV challenge material was responsible for the difference in mortality rates.

The third comparison was between the 1:10 DWV challenge group and the 1:100 DWV Aby / 1:10 DWV challenge treatment group. This tested to see if larvae receiving both antibody and challenge fared better than larvae receiving only challenge. This log-

rank comparison returned a p-value of 0.002, rejecting the null and implying that larvae receiving a 1:100 dilution of antibody in their feed in addition to the DWV challenge material had a significantly lower mortality rate than larvae receiving only challenge.

The final comparison was between the control (feed only) group and the 1:100 DWV Aby / 1:10 DWV treatment group. This comparison aimed to detect if larvae challenged with DWV and treated with antibody fared any worse than larvae receiving no challenge or antibody. This log-rank analysis returned a p-value of 0.8, failing to reject the null hypothesis and implying that larvae receiving both challenge and antibody treatment did not die at a higher rate than larvae receiving only feed in the control group.

DWV Challenge Model RT-PCR Evaluation

To confirm the mortality data on a molecular level, real-time reverse transcriptase PCR was performed on all larvae, both dead and alive, in all groups at the conclusion of the five-day DWV model. Overall Ct values for each treatment group were calculated by determining the weighted Ct value of the dead and alive larvae in that group according to the following formula: $weighted\ Ct = \frac{Ct_{dead}(\#\ larvae\ in\ lot) + Ct_{alive}(\#\ larvae\ in\ lot)}{24}$

The results are summarized in Table 3-3 and Figure 3-8.

Table 3-3. SYBR Green RT-PCR Ct Values of Larvae from DWV Model

Group	Weighted Ct Value
Control (Feed Only)	37.100
1:25 DWV Aby	37.100
1:100 DWV Aby	37.100
1:400 DWV Aby	37.190
1:10 DWV	25.270
1:25 DWV Aby / 1:10 DWV	32.951
1:100 DWV Aby / 1:10 DWV	29.229
1:400 DWV Aby / 1:10 DWV	28.525

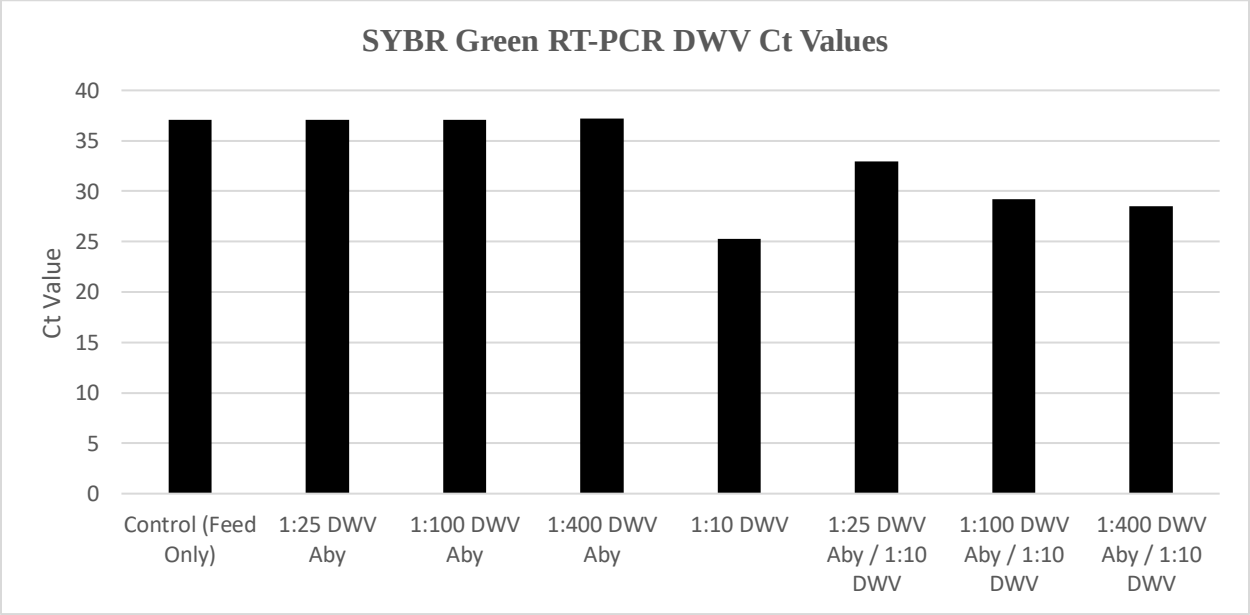

Figure 3-8. DWV RT-PCR results from DWV larval challenge model. The reported Ct values represent weighted averages of live and dead larvae pools from each experimental treatment group.

The RT-PCR results closely align with the DWV challenge model mortality data. The feed-only and antibody controls were negative for DWV, while the DWV challenge-only group returned the lowest Ct value – corresponding to the highest DWV titer. Of

the groups receiving both DWV challenge and anti-DWV IgY, the Ct value is directly proportional (and the viral titer is inversely proportional) to the volume of IgY administered to each respective group. This finding suggests that as more anti-DWV IgY is administered, more DWV viral particles are neutralized.

CHAPTER FOUR

Discussion

The results of the presented research suggest that passive pathogen-specific IgY can be effectively generated by vaccinating white leghorn chickens against various antigens and that these antibodies are entirely safe and nontoxic to *A. mellifera*. At all dosages tested in the larval trials, none of the control groups receiving Hollow Fiber Concentrated Antibody demonstrated significantly higher rates of mortality than the feed-only control group, suggesting no toxicity of the antibody in either model. Interestingly, in the DWV model, a significantly lower mortality rate was detected in the antibody controls than in the feed only controls. Given that RT-PCR showed the controls to be negative for DWV, it is not plausible for this difference to be explained by the presence of endemic DWV in the colony from which tested larvae were grafted. This unexpected finding may suggest that the administered IgY has additional benefits during development that were beyond the scope of this study.

The results also demonstrate that pathogen-specific antibodies are highly effective for the treatment of DWV and *P. larvae,* two of the most detrimental pathogens facing *A. mellifera.* Larvae receiving a 1:100 dilution of anti-DWV Hollow Fiber Concentrated Antibody in addition to DWV challenge demonstrated a statistically significant 50% reduction in mortality compared to larvae receiving DWV challenge alone. No significant difference was found between the mortality of feed only control larvae and the mortality of larvae receiving both challenge and anti-DWV IgY. This suggests that even when challenged, larvae treated with Hollow Fiber Concentrated Antibody are no more

likely to die than larvae subjected to the feed only control condition. This result was supported by RT-PCR data that reveals a clear inversely proportional relationship between the quantity of antibody administered and DWV titer.

Similar findings were derived from the *P. larvae* model. Larvae receiving a 1:100 dilution of anti-*P. larvae* Hollow Fiber Concentrated Antibody in addition to *P. larvae* challenge demonstrated a statistically significant 83% reduction in mortality compared to larvae receiving only *P. larvae* challenge. Likewise, no significant difference was found between the mortality of feed-only control larvae and the mortality of larvae receiving both challenge and anti-*P. larvae* IgY. This suggests that even when challenged, larvae treated with Hollow Fiber Concentrated Antibody are no more likely to die than larvae subjected to the feed only control condition. This result was largely supported by the CFU/mL data collected on these larvae. Among the treatment groups receiving challenge, the CFU/mL value was lowest in the group additionally receiving a 1:100 dilution of Hollow Fiber Concentrated Antibody, matching the mortality data. CFU/mL values were unexpectedly high in the treatment groups receiving challenge and either 1:25 antibody or 1:400 antibody. For the 1:400 antibody group, it was surmised that these results were due to the dosage being below the minimum protective dosage. In the 1:25 antibody group, it was speculated that elevated antibody levels led to an increased titer of antibody-antigen complex, which may have contributed to pathophysiological effects contributing to larval death and ultimately allowing for further propagation of *P. larvae* post-mortem.

Modern honeybees face a plethora of risk factors and threats to their health and ultimate survival. Of the limited options currently available for CCD prevention and/or

treatment, most are focused exclusively on the control of *Varroa.* Controlling mite levels is important, but these options alone offer limited flexibility and benefits. Given the multifactorial nature of CCD and the many pathogens that contribute to it, multipronged treatment methods are likely to be the most effective. Aside from DWV, at least 22 other honeybee viruses have been identified, and eight of these are considered to cause severe or lethal illness, including Kakugo Virus (KV), Israeli Acute Paralysis Virus (IAPV), Sacbrood Virus (SBV), Black Queen Cell Virus (BQCV), Kashmir Bee Virus (KBV), Acute Bee Paralysis Virus (ABPV), and Chronic Bee Paralysis Virus (CBPV).[60] With the exception of CBPV, all of these viruses share many conserved and consensus sequences within their genomes and have several morphological similarities such as a 20-30 nm diameter and an isometric protein capsid consisting of 60 repeated protomers that contain the VP1, VP2, and VP3 subunits.[60] All these viruses also have similar horizontal and vertical routes of transmission. Given these similarities, it seems probable that the technology described in this research could be effectively applied to these viruses as well. While these additional viruses were beyond the scope of this study, further testing is necessary to determine how broadly this technology can be applied.

This technology also has the potential to be effective in treating other species within the Apidae family, including not only other members of the *Apis* (honeybee) genus, but also of the *Bombus* (bumblebee) genus. Much like *Apis, Bombus* also plays an important role in the pollination of natural flora and is occasionally used commercially in greenhouses (usually *Bombus terrestris).*[61] *Bombus* is afflicted by many of the same pathogens as *Apis,* most notably DWV, which has the same deleterious effects on both honeybees and bumblebees. Furthermore, there is evidence that domesticated *Apis* is

responsible for spreading pathogens to native *Bombus*.[61] As domesticated honeybees continue to be used in new regions and at higher rates for commercial purposes, an increased potential arises for the transmission of viruses to the native *Bombus* species. Pathogen-specific antibody treatments could therefore benefit *Bombus* not only by possibly being effective when directly applied to *Bombus* colonies, but also by neutralizing these antibodies in the domesticated *Apis* colonies from which these pathogens are spread.

The potential for an antibody treatment specifically formulated to target the most prevalent pathogens in a given apiary or region brings with it the necessity for efficient and affordable diagnostic testing to be made available to beekeepers. Monitoring the levels of various pathogens within the herd is a common practice in the husbandry of swine, cattle, poultry, etc. due to the availability of effective treatment options. The development of similar pathogen-specific treatment options for honeybees should bring about a paradigm shift in the methodology and philosophy of beekeeping. Cost-effective multiplex PCR panels may be developed along with a means of hive sample collection (i.e. honeycomb, larval specimen, worker specimen, etc.) and transport to allow for efficient and thorough diagnostic analysis and diagnosis. Additionally, increased knowledge and understanding of the nature and mechanisms of these pathogens is likely to result from higher surveillance of hives. Metagenomic sequencing of such samples also has the potential to further reveal novel pathogens within the sampled colonies.

CHAPTER FIVE

Conclusion

Honeybee populations are in a precarious position, and the discouraging trends in their numbers and health are likely to continue without a focused effort by beekeepers to mitigate some of the factors contributing to CCD in their apiaries. While individual apiarists cannot assess factors such as reduced plant diversity and climate change on their own, they should be committed to taking steps to reduce the levels of dangerous and potentially lethal pathogens inside their colonies. DWV and *P. larvae* are currently two of the most detrimental pathogens to overall hive health and are known to initiate and/or accelerate the onset of CCD.

The production of viable, pathogen-specific IgY antibodies is possible when avian species are vaccinated for a given antigen and subsequently confer these antibodies to the yolks of the eggs they lay through passive immunity. Producing antibodies for biomedical purposes in this manner is far more cost-effective, efficient, and sustainable than many of the far more invasive methods currently employed for IgG collection. In addition to the ease with which these antibodies can be produced and concentrated, there is also mounting evidence that they are safe and effective for use in treating non-avian pathogens in non-avian species, such as DWV and *P. larvae* in *A. mellifera.*

The effectiveness with which IgY can treat pathogens in honeybees is an unexpected finding that holds lots of promise for the future of honeybee health, apiary revenues, and the overall success of the agriculture industry as it struggles to continually meet the growing global demand for food supply. The introduction of effective

treatments for DWV, *P. larvae*, and the potential for creation of IgY treatments for additional honeybee pathogens could revolutionize the future of honeybee health and beekeeping. Supplementing colonies with pathogen-specific IgY can offer the necessary protection to help compensate for honeybees' limited immune systems and ultimately help stabilize the shrinking honeybee population – cause for great encouragement and enthusiasm among apiarists and bee enthusiasts alike.

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