DISSERTATION

IVERMECTIN-TREATED BIRD FEED TO CONTROL WEST NILE VIRUS TRANSMISSION

Submitted by

Chilinh Nguyen

Department of Microbiology, Immunology, and Pathology

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Doctoral Committee:

Advisor: Brian Foy

Greg Ebel
Richard Bowen
Kathryn P. Huyvaert

ABSTRACT

IVERMECTIN-TREATED BIRD FEED FOR THE CONTROL OF WEST NILE VIRUS TRANSMISSION

West Nile virus is the leading cause of arboviral fever and encephalitis in the United States. The highest WNV disease incidence occurs along the Great Plains region of the United States, as the ecology and land use provide a supportive habitat for the main WNV enzootic and bridge vector of the region, *Culex tarsalis*. However, due to the lack of dense human population, this area often does not benefit current WNV control measures as applied by conventional mosquito control districts. Based on the ecology of WNV transmission in the Great Plains region, a strategy that targets *Cx. tarsalis* through its ornithophilic blood feeding behavior could disrupt WNV transmission. Given that the majority of *Cx. tarsalis* blood meals on the northern Colorado plains may come from doves and passerine species during the WNV transmission season, effective targeting of these or other local preferred hosts with endectocide-treated bird feed could result control of WNV transmission.

This study develops and characterizes the effects of IVM-treated bird feed in birds and biting *Cx. tarsalis* mosquitoes in both a laboratory and field setting. In Chapter 2, the effects of IVM on *Cx. tarsalis* survival were examined using both *in vitro* membrane blood meals and direct blood feeding on IVM-treated birds. Chickens and wild Eurasian Collared Doves fed solely on IVM-treated bird feed concentrations up to 200 mg IVM/kg feed exhibited no signs of toxicity, and most *Cx. tarsalis* that blood fed on these birds died compared to controls. Mosquito survivorship following blood feeding correlated with IVM serum concentrations at the time of

blood feeding, which dropped rapidly after the withdrawal of treated feed. These results suggested IVM-treated bird feed should be further explored as a hyper-localized control strategy for WNV transmission. Chapter 3 presents the development of a method to detect and quantify IVM in individual blood meals of *Anopheles gambiae* and *Cx. tarsalis,* which will be important in measuring the coverage of this intervention in the field, and accurately assessing IVM's mosquitocidal effects in field situations. This ability to detect IVM in mosquito blood meals was similar between blood fed *Cx. tarsalis* and *An. gambiae,* and between sampling times of 0 or 12 hours post blood feed. The quantity of IVM ingested in individual mosquitoes was also compared to the venous serum concentrations of live animals. Chapter 4 presents promising results from two separate pilot field trials of IVM-treated bird feed that were conducted during the summers of 2016 and 2017. Results from 2016 showed that wild birds frequently visit the IVM-treated feeders. In addition, there was an observable trend where "far" traps that are expected to be beyond the zone of control had more WNV-positive pools compared to "near" traps at both ELC and ARDEC South sites. Results from the 2017 study continued to be promising, where birds were again visiting IVM-treated feeders and IVM could be detected in the sera of birds sampled by IVM feeders. There was also a trend of higher VI for the control sites compared to IVM sites for the 2017 season.

The efficacy of IVM-treated bird feed was evaluated in two pilot trials where natural WNV transmission cycles occurred in wild birds and *Cx. tarsalis,* but should be followed up with field seasons with many control and IVM sites to allow for a robust analysis of IVM effects. This study introduces the novel concept of using systemic endectocides for controlling WNV transmission, and this concept could be explored for other arboviruses.

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1.1 West Nile virus

1.1.1 West Nile virus background

West Nile virus is within the family *Flaviviridae* and genus *Flavivirus*. West Nile virus is an enveloped virus with an approximately 50 nm diameter, a host-derived lipid bilayer membrane, and a single-stranded positive-sense RNA genome of approximately 11 kilobases (1). This RNA genome encodes for 3 structural proteins and 7 non-structural proteins in the following order: capsid-membrane-envelope-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5. The open reading frame for these proteins follows a 5' non-coding region and a 3' non-coding region follows the NS5-3 (1). Viral replication occurs in the cytoplasm in close association with the rough endoplasmic reticulum (ER), viral assembly follows in the ER lumen, followed by release (1).

West Nile virus belongs to the Japanese encephalitis virus (JEV) serocomplex, which also includes St. Louis Encephalitis virus (SLEV) and Murray Valley virus (1, 2). While there are at least 5 phylogenetic lineages for WNV, only lineages 1 and 2 have been associated with significant human outbreaks (3). Viruses isolated from the western hemisphere, Africa, the Middle East, and Europe represent lineage 1a, Kunjin virus from Australasia is lineage 1b, and lineage 1c constitutes viruses from India (3, 4). The first North American isolates of WNV from the New York 1999 outbreak were most closely related to a lineage 1a virus isolated from a goose in Israel in 1998 (5), and this genotype has largely been displaced by a new genotype (WN02) that may have adaptations to enhance transmission in North American mosquito vectors (6, 7).

West Nile virus was introduced into the United States in 1999 and detected during an outbreak of meningoencephalitis in New York City (1, 2, 8). Initial human cases were originally misidentified serologically as SLEV. Concurrent bird deaths from WNV were identified throughout New York City, including the Bronx Zoo, indicating potential bird-mosquito viral amplification (9). During 1999-2000, 78 meningoencephalitis cases were detected within the greater New York City metropolitan area with onsets in August and September (10, 11). However, the emphasis on neuroinvasive disease during surveillance efforts likely resulted in an underestimation of WN fever cases, further evidenced by a post-epidemic serosurvey of New York City that estimated roughly 100 asymptomatic WN viral infections and 20 WN fever cases for each WN meningoencephalitis case (12). Within four years of this first introduction, WNV had been detected in all 48 contiguous states, parts of southern Canada, Mexico, and parts of Central and South America (9).

Since its original introduction in 1999, West Nile virus has become the leading cause of domestically acquired arboviral disease in the United States (13, 14). There have been upwards of forty thousand reported cases between 1999 to 2015 (15); however, it is suspected that this number vastly underreports the number of infections, given the asymptomatic nature of most WNV infections, and the number of infections may actually be upwards of 3 million (16). Based on data extrapolated from Colorado patients diagnosed with multiple manifestations of WNV in 2003 to national WNV surveillance data, it is estimated that the cumulative cost of WNV hospitalized cases from 1999 to 2012 was $778 million (17).

Because 80% of WNV infections are asymptomatic (10), most WNV infections go unreported, resulting in an underestimation of the number of infections. The greatest proportion of symptomatic infections are cases of West Nile fever, consisting of generally flu-like

symptoms such as myalgia, headache, rash, and arthralgia (1, 18). Most patients diagnosed with West Nile fever have a complete recovery, although the fatigue and malaise may linger for multiple months (2). Less than 1% of infections result in a neuroinvasive form of disease, manifesting as meningitis, encephalitis, or acute flaccid paralysis (19). WN meningitis is the least severe manifestation of the neuroinvasive cases, and like West Nile fever, often results in a favorable outcome with persistent fatigue and myalgia (19). Elderly and immunocompromised persons are at highest risk for West Nile encephalitis, which ranges from self-limited confusion to severe encephalopathy, coma, and death (19) Neuroinvasive cases often resolve with neurological sequelae and predictably have higher case fatality rates than non-neuroinvasive cases (19).

1.1.2 West Nile virus incidence in the United States

West Nile virus cases have been reported in all 48 contiguous states. The highest incidence of WNV disease occurs in the Great Plains region of the United States (14, 16, 20). The five states with the highest state-specific WNV incidence in descending order are South Dakota, Wyoming, North Dakota, Nebraska, and Colorado (16). The high incidence in this region is linked to the main vector in this region, *Cx. tarsalis*, which is a highly efficient vector of WNV that readily blood feeds on humans in the mid-late summer and is highly abundant along the riparian corridors and edges of water reservoirs within this region (20, 21). Furthermore, the use of irrigated farmland in this region provides a suitable and supportive habitat for large numbers of *Cx. tarsalis* (22, 23). However, given the heterogeneous nature of WNV ecology, this association between WNV incidence and irrigated agriculture is not evident across the entire Great Plains region. Chuang et al. examined landscape-level spatial patterns for

WNV risk in disparate areas of South Dakota and did not find irrigated agriculture to be a predictor of WNV incidence (24).

1.1.3 West Nile virus ecology

West Nile virus is maintained in an enzootic cycle between viremic birds and ornithophilic *Culex* species mosquitoes, and this interaction of viremic avian hosts and *Culex* spp. has important implications for WNV ecology. The contribution of a particular bird species to WNV transmission depends on the level of vertebrate reservoir competence as well as the proportion of mosquito blood meals derived from the species. However, ornithophilic *Culex* mosquitoes have specific blood meal preferences among bird sources. This preference for certain bird species determines the level of contact between vectors and important vertebrate reservoirs, and therefore, examining avian abundance without considering mosquito blood feeding preference is a poor measure of a species' contribution to WNV transmission (25). It is generally considered that more ornithophilic mosquitoes serve as amplification vectors that drive enzootic transmission within mosquitoes and birds, while more catholic feeding mosquitoes serve as bridge vectors to drive epidemic transmission of WNV to humans and other dead-end mammalian hosts (26).

Bird species vary widely in their reservoir competence, measured using an index that reflects the relative number of infectious mosquitoes that would result from feeding on a bird species, as a function of viremia following mosquito-borne infection. This reservoir competence value is the product of susceptibility (the proportion of birds that become infected following exposure), mean daily infectiousness, and the duration of infectiousness (27). Following experimental infection of wild birds and using a threshold infectious viremia of 10^5 PFU/mL serum, birds with the highest level of reservoir competence were passerine birds (27). Generally,

passerine and charadriiform orders had the highest viral titers with the longest duration, while psittacine and gallinaceous birds had the lowest viral titers with the shortest viremia (27). A study looking at the reservoir competence of Eurasian collared doves *(Streptopelia decaocto)* following experimental infections found low viremia levels suggesting that they are not an important amplifying host for WNV (28). However, the authors do suggest that these doves could play an important role where they are locally abundant (28), confirming that the role of a bird species in WNV transmission depends on both reservoir competence and contribution to mosquito blood meals.

West Nile virus transmission in North America varies regionally according to important mosquito vectors and avian hosts. Intense and focused feeding on highly infectious and viremic birds such as robins by both *Cx. pipiens* and *Cx. tarsalis* in the early summer months contributes to periods of intense WNV amplification, while a subsequent decrease in the availability of robins due to dispersal after breeding results in a feeding shift towards mammals and other humans (29). These feeding shifts synergistically contribute to human WNV infection; focused feeding on highly infectious and viremic birds results in high numbers and proportions of WNV-infectious mosquitoes, whereas feeding on humans at a high rate during the early summer would result in blood meals that do not contribute to WNV-infectious vectors (29).

Given regional differences in important mosquito vectors and avian reservoirs, WNV transmission cycles should be analyzed with respect to geographical distinctions. Blood meal sources of WNV relevant vectors of the northeastern United States, including *Cx. pipiens*, *Cx. salinarius*, and *Cx. restuans*, were identified (30). Both *Cx. pipiens* and *Cx. restuans* were highly ornithophilic with 93.1% and 100% avian-derived blood meals, respectively, and the largest proportion of these avian blood meals were from robins. The majority of *Cx. pipiens* avian-

derived blood meals were from highly competent WNV reservoirs with 37.7% from robins, 10.4% from gray catbirds, and 9.9% from house sparrows. Additionally, there was a seasonal shift in *Cx. pipiens* feeding habits with a statistically significant decrease in the number of robin-derived blood meals from June through October (30). *Cx. salinarius* displayed more opportunistic feeding habits with 36% of blood meals containing only avian blood, 53% only mammalian, and 11% containing a mix of avian and mammalian blood. Human-derived blood meals were found in 1 *Cx. pipiens* and 2 *Cx. salinarius*. From these seasonal blood meal preferences, it was concluded that ornithophilic *Cx. pipiens* and, particularly, *Cx. restuans* serve as important enzootic amplification vectors of WNV, especially during the early WNV season. *Cx. salinarius* was determined to also be an important bridge vector that transmits WNV from viremic birds to mammalian hosts.

This WNV transmission cycle differs in California when the blood meal sources as well as WNV-infection status of different mosquito vectors in southern California were considered by Molaei et al (30). *Culex quinquefacsiatus* was considered as an important WNV vector, as it contributed nearly 80% of WNV-positive mosquito pools from sampling efforts, and its blood meals from both important amplifying passeriform birds and mammals implicate *Cx. quinquefacsiatus* as both an amplification and bridge vector (31). Although *Cx. tarsalis* has been implicated as a highly competent WNV vector, it likely plays a secondary role in transmission as compared to *Cx. quinquefacsiatus* in this region. While *Cx. tarsalis* displayed opportunistic feeding with a preference for birds that implicated it as a potentially important epidemic and epizootic vector, it is temporally and spatially constrained. Additionally, *Cx. tarsalis* displayed lower infection rates as compared to *Cx. quinquefacsiatus*, with seasonal maximum likelihood estimation (MLE) of virus infection ranging from 0.3 to 2.75 per 1000 mosquitoes as compared

to a range of 0.59 to 9.52 per 1000 mosquitoes in *Cx. quinquefacsiatus*. All *Culex stigmatosa* blood meals were obtained from avian sources, and this ornithophilic feeding behavior has been previously reported (31, 32).

In the western Great Plains region, there is evidence that *Cx. tarsalis* is the primary vector of WNV, particularly in non-urban areas (22). There is a close correlation between the weekly vector index (VI), the product of species abundance per trap night and the proportion of infected mosquitoes, of *Cx. tarsalis* and the number of human WNV disease cases, with a 1-2 week lag between the peaks of VI and number of human disease cases (33, 34). Additionally, there is a strong association between *Cx. tarsalis* abundance and human WNV disease cases with a longer delay of 4-7 weeks between peak mosquito abundance and human disease onset. Furthermore, *Cx. tarsalis* were found in higher abundances with higher WNV infection prevalence than *Cx. pipiens* along a riparian corridor in eastern Colorado containing both prairie and montane habitats (35). Seasonal blood meal sources of *Cx. tarsalis* have been characterized in the western Great Plains region, with avian hosts preferred to mammalian hosts throughout the WNV transmission season (36, 37). As in other regions, robins serve as important early-season amplifying WNV hosts, contributing an estimated 60 to 1% of the WNV-infectious *Cx. tarsalis* with the proportion declining between June and August (36). This shift from robins as a blood meal source as the summer progresses also coincides with a gradual increase in mammalian blood meal sources over the same season (36). Doves serve as important blood meal sources throughout the summer and contribute 25-40% of the WNV-infectious mosquitoes per month (36).

2.1 Insecticides and Other Vector Control Methods

2.1.1 West Nile virus control

West Nile virus control efforts greatly vary by district and consist of vector-focused management. Mosquito control districts generally focus on mosquito population management and public education efforts. Mosquito population control can focus on source reduction to target larvae in their aquatic habitats, or controlling adults with adulticiding when certain population thresholds are met.

Larviciding is generally considered less environmentally damaging and more cost-effective than spraying of adulticides due to the more efficient targeting of mosquitoes (8, 38). Commonly used larvicides include *Bacillus thuringiensis, Bacillus spaericus,* methoprene, and oils (39). For WNV control, *Culex* spp. are often specifically targeted using applications of larvicides to stormwater catch basins (40). For example, one study modelling the impacts of a single yearly application of bacterial larvicide (*B. sphaericus)* over a seven year period found larvicide application reduced the odds of finding *Culex spp.* larvae by a factor of 7 (41). However, studies have shown that multiple variables may contribute to reduced efficacy of these larvicides. For example, monitoring of larval catch basins following an application of an extended-release spinosad tablet showed that 76.8% of the basins were missing tablets 1-17 weeks following application, potentially due to rapid dissolution or flushing, and there was no observed reduction in mosquitoes (42). Further monitoring for efficacy of multiple larvicides within catch basins in the Chicago area also indicated that over a quarter of the catch basins may not be adequately protected by the recommended single application (43). Another study evaluating multiple larvicides in both regular and extended duration formulations found at least 48% reduction in total seasonal pupal numbers compared to untreated basins, but also found

considerable variation in effectiveness, regardless of regular and extended regulation formulas (44). These studies indicate that larvicide applications can be effective in reducing mosquito abundance, but that many variables may reduce larvicide effectiveness.

To target adult mosquitoes, aerosolized chemical insecticides are sprayed into the air, either through aerial spraying using aircraft or truck-mounted ultra-low volume spraying. Previous studies have demonstrated that aerial spraying is effective in reducing target mosquito populations (45–48). Furthermore, an evaluation of aerial spraying of pyrethrin in the Sacramento area reduced the number of human WNV cases in the treated area as compared to the untreated area, with the odds of WNV infection 6 times higher in the untreated area (47). Macedo et al. also found significant reductions in the abundance of both *Cx. pipiens* and *Cx. tarsalis* as well as the infection rate of *Cx. tarsalis* following an aerial application of pyrethrin and piperonyl butoxide, analyzed by pre- and post-trapping both inside and outside the spray zone (48). However, the literature for ground ULV applications generally indicates that adulticides applied in this manner can be effective at reducing mosquito populations (45), but may not directly impact viral transmission (49–51). Evaluation of sequential ground applications of pyrethrins and piperonyl butoxide demonstrated that vector abundance was reduced, but virus transmission was unaffected and WNV continued to disperse throughout the Coachella Valley of California; the timing of the applications which were started 1 month after the initial detection of virus may have influenced its effectiveness in reducing WNV transmission (51). Another study in the Chicago area demonstrated that ground ULV treatments using sumithrin decreased *Culex* mosquito abundance significantly, but MLE of infection rates varied independently of adulticide treatments, suggesting that there was no direct effect on MLE from adulticide use (49).

While these current vector control efforts are important, they also pose many challenges. For example, the use of larvicides and adulticides often faces community opposition due to perceived environmental and health risks. While studies have provided evidence that mosquito adulticiding efforts are safe for humans and outweigh the risks presented by WNV (52), there have been studies linking exposure to pesticides commonly used for mosquito control with adverse human health effects (53–55). Pesticides may also pose a risk to other off-target animals (56, 57). Furthermore, these vector control efforts generally rely on mosquito control districts, which do not exist within all communities and also vary significantly based on their individual funding levels and community characteristics. For example, local politics of mosquito control and subsequent differences in vector control policies may have contributed to local-scale geographical variation in WNV incidence within the Chicago area (58). Additionally, there is unequal funding across mosquito control programs, with urban ones receiving funding at a higher rate than rural mosquito control programs (59, 60). This corresponds with rural areas with a higher incidence of WNV cases receiving less protection from WNV relative to urban areas with lower WNV risk (22, 61).

2.2.1 Systemic endectocides

One innovative class of insecticides currently being studied is systemic endectocides. Systemic endectocides are given to a target host and vector species are targeted through their interactions with the treated host, often through blood feeding on the host.

Rodent baits with feed-through and systemic insecticide activity have been evaluated to control the phlebotomine sand fly vectors of zoonotic cutaneous leishmaniasis and visceral cutaneous leishmaniasis. A wide variety of insecticides including fipronil, diflubenzuron, eprinomectin, ivermectin, methoprene, pyriproxyfen, and novularon have been tested in rodent

models such as Syrian hamsters (*Mesocricetus auratus*), as well as more epidemiologically-relevant rodents such as *Meriones shawi* and *Bandicota bengalensis* to control multiple species of phlebotomine sand fly vectors (62–66). Derbali et al. assessed fipronil-treated rodent bait in *Meriones shawi* as a systemic and feed-through insecticide to control *Phlebotomus papatasi* in both laboratory and field settings (67). In laboratory conditions, there was significantly increased mortality in *P. papatasi* larvae fed on feces of fipronil-diet *M. shawi* as well as adult females bloodfed on *M. shawi* fed a fipronil-containing diet as compared to larval and blood feeding bioassays using control-diet *M. shawi* (67). In field trials where a single application of fipronil-treated or untreated bait was placed in front of active rodent burrows, there was an 80% reduction in *P. papatasi* populations at the fipronil-treated site compared to the untreated site for up to 6 weeks following a single bait application (67). A field trial assessing a single application of imidacloprid rodent bait in *M. shawi* burrows also resulted in 90% reduction in *P. papatasi* populations for up to 4 weeks, as compared to untreated rodent bait (68).

In addition to phlebotomine vectors, acaricides delivered through baiting strategies have been applied to the mammal reservoirs of Lyme disease to target tick vectors. Examples include oral endectocide-treated baits targeted to definitive *Ixodes* hosts and passive application of topical acaricides upon consumption of treated bait. Systemic endectocides delivered in oral baits have been formulations of ivermectin (69, 70), and these studies will be discussed in a later section. One passive application study was conducted in a coastal island community in Connecticut to evaluate the use of bait stations targeted to white-footed mice, *Peromyscus leucopus*, where bait boxes topically applied fipronil to mice during consumption of bait (71). Following three years of treatment, there was a significant reduction in multiple parameters including significantly reduced nymphal and adult tick infestation as well *B. burgdorferi*

infection in *P. leucopus* (71). In addition, there were significantly fewer questing adults and host-seeking nymphs, both with reduced *B. burgdorferi* infection rates, at treatment baited sites as compared to control, untreated sites (71). Most studies of topical acaracide application to deer utilize a "4-poster" United States Department of Agriculture-Agricultural Research station-patented application device where the head, neck, and ears of deer come into contact with acaricide-treated rollers upon feeding on bait (72). One study conducted an evaluation of 2% armitraz in these 4-poster devices, comparing control and treatment pastures holding *Odocoileus virginianus* (73). Following three consecutive years of armitraz application, there was a significant reduction in the number of adults and nymphs of 94% and 92%, respectively, compared to the untreated pasture (73).

Another variation on this targeted strategy is the use of antibiotic-treated bait to prophylactically target reservoirs of vector-borne bacteria and reduce the bacterial infection rates in arthropod vectors. For example, there was field trial conducted using doxycycline hyclate-impregnated bait to prophylactically treat rodent reservoirs and consequently reduce infection prevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in *Ixodes scapularis* ticks (74). Following two years of treatment, *B. burgdorferi* and *A. phagocytophilum* infection rates were reduced by 86.9% and 74%, respectively, in small mammals and 94.3% and 92% in questing *Ixodes* ticks, indicating this could be an effective method to reduce infection rates within tick vectors (74).

3.1 Ivermectin

Ivermectin (IVM) is a widely-used antiparasitic drug, with broad spectrum activity, high efficacy, and a wide margin of safety (75). It is a chemical derivative of avermectin B_1, or abamectin, which is produced by *Streptomyces avermitilis*, a soil actinomycete (75). It has a

wide range of uses in the livestock industry, companion animal care, and treatment of parasitic diseases in humans (75, 76). IVM kinetics can vary widely with formulation and administration method, but are generally characterized to have a slow absorption and excretion, wide tissue distribution, and low metabolism across multiple species (75).

3.1.1 Ivermectin pharmacokinetics

Characterization of IVM pharmacokinetics in humans is primarily in relation to onchocerciasis control. For onchocerciasis treatment, IVM is given at an oral dose of 150 μg/kg, one to three times yearly (76). There were no significant pharmacokinetic/pharmacodynamic differences between healthy and microfilaria-positive volunteers given IVM; both groups had a t_{max} (time to maximum concentration) of about 4 hours (77). Additionally, both healthy and microfilaria-positive groups displayed a secondary peak of IVM in their plasma following an initial decrease, indicating likely enterohepatic recycling from IVM excretion in bile (77). Furthermore, both healthy and microfilaria-positive groups displayed similar distributions of IVM following absorption, with the highest concentrations in fat and the lowest in subcutaneous fascia, which is expected given IVM's lipophilic nature (77). IVM has also been characterized in human plasma, with strong binding to plasma proteins and serum albumin (78). Cytochrome P4503A4 in liver microsomes is responsible for IVM metabolism (79), and IVM is excreted primarily in feces (76). Identified plasma metabolites of IVM are less polar than IVM and could be fatty acid ester conjugates of the monosaccharides or aglycone of the parent drug (80). Excreted metabolites include 3″-*O*-desmethyl-H_2B_{1a} in urine and 3″-*O*-desmethyl-H_2B_{1a}-Monosaccharide in feces (80).

There have been many studies evaluating pharmacokinetic parameters of IVM in humans at different doses and dosing schedules. Plasma $t_{1/2}$ (elimination half-life) has been estimated to

range from 12 to 56 hours (81–84). Reported plasma maximum concentration (C_{max}) was about 20 ng/mL for a 6 mg dose, ranged from 23.5 ng/mL to 81 ng/mL for a 12 mg dose, and was between 33.8 to 54.4 ng/mL for a 150 µg/kg dose (76, 77, 81, 85, 86). T_{max} reported for 12 mg doses ranged from 3.4 to 10.3 hours, which was more variable than the 4.3 and 4.7 hours reported for 6 mg doses (81, 85, 86). A study evaluating different oral IVM formulations found significantly increased area under the plasma vs time curve (AUC_{PK}) for a 12 mg dose given in a ethanolic solution (1473 ng h/mL) or capsules (1034 ng h/mL) as compared to tablets (885 ng h/mL) (86). IVM given in an ethanol-based solution also had twice the systemic bioavailability of IVM given in a tablet or capsule formulation, which may be explained by differences in IVM solubility, although all three forms had a similar rate of absorption (86).

Ivermectin pharmacokinetics is best characterized in cattle, where IVM is routinely administered for control of various parasites. Various IVM administration methods for cattle include subcutaneous (SC), intramuscular (IM), oral, and topical routes. The subcutaneous route is most extensively studied, and the results are highly variable depending on factors such as cattle breed, body condition, IVM formulation, IVM quantification method and analysis (75). Across these SC studies, C_{max} values were generally above 20 ng/mL with a T_{max} of at least 24 hours and an AUC above 150 ng day/mL for plasma (75). One study specifically characterized IVM tissue distribution relative to common parasite tissue tropisms in cattle following subcutaneous injection of 200 µg/kg (87). IVM was detected at concentrations greater than 0.1 ng/mL for up to 48 days post-treatment in each of the tissues and fluids examined – plasma, abomasal and small intestinal fluids and mucosal tissues, bile, feces, lungs, and skin – and up to 58 days in bile and feces (87). While most tissues had higher concentrations of IVM as compared to plasma, there was a high correlation between plasma and tissue concentrations (87).

Pharmacokinetic parameters for bile and feces, the substrates where IVM was longest-lived, were the following: T_{max} of 1 day (both), C_{max} of 164 ng/mL (bile) and 115 ng/g (feces), AUC of 1663 ng day/mL (bile) and 1193 ng day/g (feces), and a maximum retention time (MRT) of 6.2 days (bile) and 6.3 days (feces) (87). While there was no difference observed in plasma availability between SC and IM administration, increased blood flow in muscle tissue may account for the delayed $T_{1/2}$ and T_{max} observed for SC administration (88). Topical IVM is complicated by licking behavior of cattle. Laffont et al. found homogenous IVM plasma clearance (270±57.4 mL/kg/day) among 12 cattle given 200 μg/kg IVM subcutaneously (89). However, when these calves were given 500 μg/kg IVM topically, and separated into a licking and non-licking group, there were differences in pharmacokinetic parameters. While plasma $t_{1/2}$ was similar between topical and IV administration for the licking group, the plasma $t_{1/2}$ was significantly longer for topical application as compared to IV administration in the non-licking group(89). Furthermore, topical systemic IVM availability was higher and more variable for the licking group compared to the non-licking group (89). This demonstrated that oral absorption of IVM through calf licking behavior should also be considered during IVM topical application. Characterized IVM liver metabolites found in cattle include 24-OH-H_2B_{1a} and 24-OH-H_2B_{1b} (90). In addition, nonpolar metabolites have been identified in fat tissue, suggesting that these are liver metabolites that have become esterified with fatty acids and stored in fat (91).

Because IVM use in birds is primarily off-label, the pharmacokinetic profile of IVM in various bird species is not well-characterized. Moreno et al. characterized the pharmacokinetics, metabolism, and tissue profiles of IVM in laying hens *(Gallus gallus)* using IVM intravenous (IV) and oral routes. For a single IV administration of 400 μg/kg, IVM was quantified in plasma with the highest concentration (739.6±50.2 ng/mL) 30 minutes after administration and the

lowest concentration (0.38±0.06 ng/mL) 10 days following, demonstrating an expected IV pharmacokinetic profile (92). For daily oral IVM treatment of 400 μg/kg over 5 days, the tissues with the highest concentrations of IVM were liver, followed by skin and fat, kidney, plasma, then muscle, which is consistent with the lipophilic nature of IVM (92). As expected, concentrations were highest on the first day post-treatment and lower than those from IV administration (92). Further consistent with IVM's lipophilicity, significant levels of IVM were found in the yolks of the eggs laid by hens receiving oral IVM, but not detected in egg white (92).

Bennett et al. 2012 demonstrated transfer of IVM through crop milk of pigeons (93). Adult pigeon pairs were given an estimated dose of 21.4 μg/kg of body weight per day in drinking water three days, and IVM was quantified in brooding squabs co-housed with the adult pigeons. At the end of the IVM treatment period, there was no difference in the IVM amount quantified in squab pigeon breast as compared to liver, contrasting previous findings (92), although the amount of subcutaneous fat attached to breast tissue was variable (93). IVM was no longer found in detectable levels in squab breast or liver tissue 1 week post IVM-dosing (93). This contrasts findings from Miller et al., in which broiler poultry given IVM-treated feed at 2 μg/g for 5 weeks did not have detectable IVM liver residues, even without a withdrawal period (94).

3.1.2 Ivermectin detection and quantification

The widespread use of IVM and other avermectins across industries has led to the development of multiple detection and quantification methods. Many of these methods are focused on the veterinary use of IVM. Examples include characterizing pharmacokinetic and

pharmacodynamic profiles of IVM in various animals, particularly cattle and swine, as well as examining elimination of IVM in various matrices such as milk.

Because of IVM's widespread use, many methods for extraction of IVM and other related avermectins have been developed. Examples of matrices for which extraction methods have been developed include plasma of various animals, cow milk, cattle feces, veterinary drugs, and beetle carcasses (78, 95–101). One of the simplest methods is a cold methanol precipitation, optimized for bovine plasma (102). Solid phase extraction (SPE) is also commonly used as it can optimally remove interfering constituents present in the matrix, particularly when paired with appropriately selective eluting solvents (95). However, SPE is time-consuming, requires multiple steps which may affect reproducibility, introduces sample loss during evaporation, and potentially introduces contamination (103).

One of the simplest ways to quantify IVM is using UV detection following extraction and reverse phase high performance liquid chromatography (HPLC). This is based on the molecular structure of IVM, which has a conjugated-diene chromophore with a UV absorbance maximum at 245 nm (95). One study applying this HPLC-UV method to IVM in pre-mixed animal feed found a detection limit of 6 mg/kg, with a ±3.4% mean relative error with <3% precision across the range of 50-150 mg/kg (104). Another study evaluating the use of HPLC-UV quantification for IVM in milk had a mean recovery of about 80% with 3.2% interassay coefficient of variation (99). While the simplicity of this assay may lend itself to higher reproducibility, one significant disadvantage is the lower sensitivity, ranging from 1-2 ng/mL, compared to other methods (81, 95).

Combining HPLC with fluorescent derivatization of IVM and fluorescent detection increases the sensitivity of the method significantly (95). Fluorescent detection can be performed

at 365 nm (excitation) and 475 nm (emission) (102). Fluorescent detection requires derivatization of IVM to produce a fluorophore before injection into the HPLC system. One common derivatization utilizes *N*-methylimidazole (MI) and trifluoracetic anhydride (TFAA) as the derivatization reagent (95, 102). While this fluorescent derivatization reaction is widely practiced, the unstable nature of the derivatization product requires a system where the in-line derivatization can immediately proceed injection and analysis in the HPLC instrument, limiting the application, throughput, and reproducibility of this method. The limitations of this fluorescent derivatization reaction were further characterized by Berendensen et al. (105). The derivatization reaction using MI and TFAA results in two fluorescent derivatized products present in equilibrium, a fluorescent aromatic derivative containing a free hydroxy group at the glycosidic ring (flu-OH) and an acetyl derivative that is a tri-fluoracetyl ester (flu-TFA) (105). This reaction occurs for avermectins with the appropriate reactive group – an identical dihydroxylated tetrahydro benzofuran ring that can be converted into a chromophoric group. This is based on the *in situ* formation of an acylating reagent through the reaction of MI and TFAA, which can further react with the hydroxylic groups on avermectins. In the presence of a strong nucleophilic base such as MI or triethylamine (TEA), a double deacetylation reaction of the benzofuran ring will occur, resulting in strongly fluorescent aromatic derivatives (105). Berendensen et al. optimized a derivatization method to preferentially synthesize a stable flu-TFA derivative, circumventing many of the issues with the previous derivatizations that result in an unstable flu-TFA derivative converting into flu-OH or a mixture of the two derivatives, which can complicate the analysis (105).

Beyond HPLC-UV or HPLC-fluorescence approaches to IVM quantification, methods utilizing mass spectrometry have also been developed (101, 102, 106). Advantages of these

techniques include potential increases in sensitivity, potentially increased reproducibility due to automation and no need for derivatization, and additional capability for further structural characterization. Additionally, the lack of fluorescent derivatization needed for mass spectrometry allows for simultaneous high-throughput analysis of multiple drugs within the same matrix (107).

3.1.3 Ivermectin as an endectocide for vector control

IVM as an endectocide to control vector-borne disease is perhaps best characterized for disruption of malaria transmission. IVM is currently given in mass drug administrations (MDA) to control and treat onchocerciasis and lymphatic filariasis, and many of these MDAs occur in malaria-endemic areas. Given the co-endemicity of onchocerciasis and malaria, there is a unique opportunity to simultaneously target both diseases, as the IVM therapeutic dose for onchocerciasis also results in concentrations of IVM in blood that are lethal to multiple vectors of malaria including *Anopheles gambiae, Anopheles farauti,* and *Anopheles punctulatus* in laboratory settings (108–110). This leads to the idea that IVM MDA for onchocerciasis could also disrupt malaria transmission, as the IVM blood concentration would also result in lethal blood meals for these anthropophilic vectors of malaria. Because the effect of IVM on mosquito mortality is directly related to the time that there is a lethal IVM concentration in blood, the lethal effect of IVM could be a function of AUC_{PK}. A theoretical LC_{100} would kill 100% of biting mosquitoes, and therefore the efficacy of IVM would be a function of the AUC that is below the LC_{100}. This is supported by modelling that demonstrates that the length of time that IVM is above mosquitocidal levels is a critical component affecting impact on malaria transmission (111).

However, there are also important sub-lethal effects of IVM in mosquitoes that could additionally affect malaria transmission, making the later end of the AUC_{PK} important even as the concentrations of IVM decrease below lethal levels. Studies have observed diminished fertility in mosquitoes imbibing a sub-lethal dose of IVM and a reduced hatching rate for the eggs laid by IVM-fed mosquitoes (109, 112). In addition, there are important fitness effects of sub-lethal IVM including knockdown, decreased flight ability, and decreased re-blood feeding frequency (110, 113). There is also evidence of IVM inhibition of plasmodium sporogony (114). All of these IVM sub-lethal effects would contribute to reduced vectorial capacity for *Plasmodium* transmission and amplify the effect of IVM MDA for decreasing malaria transmission.

This concept of IVM administrations affecting mosquito populations has also been demonstrated in field settings. In southeastern Senegal, resting mosquitoes were aspirated from homes in treatment villages that were given an IVM MDA and paired control villages without a MDA; mosquitoes were sampled pre-MDA, 1 to 6 days post-MDA, and 7 or more days post-MDA (115). Following aspiration, mosquito species were identified and mosquitoes were held to monitor survival. There was a significant reduction in survivorship for both *An. gambiae* and *Anopheles arabiensis* from treated villages as compared to control villages. The effects were strongest in *An. gambiae,* where those caught from treated villages 1-6 days post MDA still had reduced survival, including when compared to mosquitoes collected pre-MDA and more than 7 days post-MDA. One explanation for the stronger effect in *An. gambiae* as compared to *An. arabiensis* is that 97.8 % of the *An. gambiae* tested had blood fed on humans, while only 75% of *An. arabiensis* blood meals tested were from a human source (115).

This potential use of IVM as a systemic endectocide to disrupt malaria transmission has been further explored in the diverse environments of Senegal, Liberia, and Burkina Faso (116). MDA consisted of 150 µg/kg IVM in Senegal for onchocerciasis treatment, and 150 µg/kg IVM plus 400 mg albendazole for treatment of both onchocerciasis and soil-transmitted helminths in Liberia and Burkina Faso. MDA coverage in treatment villages was 82.1-84% in Senegal, 76.2% in Liberia, and 83% in Burkina Faso (116). *An. gambiae* survivorship, parity rate, and sporozoite rates were analyzed between treatment and control villages using indoor-resting, bloodfed mosquitoes aspirated from within houses; mosquito sampling was done both pre- and post-MDA. A 33.9% reduction in *An. gambiae* survivorship was observed for one week following MDA. Longer-lasting effects were seen in parity rates and sporozoite rates, where significant reductions in these variables lasted for 2 weeks or more (116). This longer-lasting reduction in parity rate, which represents a shift in the age structure of the mosquito population towards younger mosquitoes that are unable to transmit malaria, is an important component of this malaria transmission control strategy.

IVM-treated feed given to white-tailed deer (*Odocoileus virginianus)* has also been explored as a method to control *Ixodes scapularis,* the definitive vector of Lyme disease. *O. virginianus* that populated a small, uninhabited Maine island were provided IVM-treated corn for 5 consecutive spring and fall seasons (70). The treatment effect was evaluated by observing adult tick numbers collected from deer, as well as the number of ticks that sufficiently engorged for successful oviposition, and their subsequent success of oviposition, and the rates of larval eclosion from laid eggs. These parameters were significantly reduced in ticks that engorged on 8 of the 16 deer that had IVM serum levels quantified equal to or above 15 ng/mL. However, only a minority of the deer had target IVM serum levels, possibly due to specific deer dominance at

feeders, seasonal availability of alternate food sources, and an underestimation of the deer population when providing IVM-treated feed (70).

IVM has been used as systemic endectocide to control tick populations generally, without respect to vector-borne disease. In one example, IVM-treated corn was evaluated in white-tailed deer in a treatment pasture as compared to untreated deer in a control pasture (69). Subsequent *Amblyomma americanum* collections showed a reduction of 83.4% in adults, 92.4% in nymphs, and 100.0% in larvae in treatment compared to control pastures (69). Lancaster et al. also demonstrated systemic efficacy of SC IVM administration of cattle against *Am. americanum* (117). One study evaluated efficacy of IVM against *Boophilius microplus* in a single pour-on application as compared to a double treatment application with a 4 day interval between treatments and observed comparable reductions in tick number and reduced weights for engorged females and egg mass weights for treated cattle (118). In addition, Miller et al. demonstrated that the use of a slow-release bolus application of IVM resulted in a reduction in the free-living *Boophilus annulatus* on untreated sentinel cattle pastured with the IVM-treated cattle as compared to sentinel cattle pastured with untreated control cattle (119). These deer and cattle studies demonstrate the potential of IVM-treated bait as a systemic endectocide to target vector populations, including with the goal of disrupting vector-borne disease transmission.

It is important to note that not all uses of systemic endectocides have the same end point. The development of systemic endectocides for controlling mosquito-borne disease transmission is highly novel as the goal is to reduce the number of infectious mosquitoes before they are able to transmit disease, without a focus on reducing the entire population numbers. This is seen with an important shift in the population age structure of *An. gambiae* following IVM MDA from older, parous mosquitoes that have had an opportunity to become infectious, towards younger,

non-infectious mosquitoes (116). This differs from the above studies targeting tick populations using IVM and the previously reviewed studies of systemic endectocides where the focus is generally on reducing total population numbers.

4.1 Research rationale and summary of aims

The highest WNV disease incidence occurs along the Great Plains region of the United States (120), as the ecology and land use provide a supportive habitat for the main WNV enzootic and bridge vector of the region, *Culex tarsalis* (22). However, due to the lack of dense population, this area often does not benefit current WNV control measures as applied by conventional mosquito control districts (61). Based on the ecology of WNV transmission in the Great Plains region, a strategy that targets *Cx. tarsalis* through its ornithophilic blood feeding behavior could disrupt WNV transmission. Given that the majority of *Cx. tarsalis* blood meals on the northern Colorado plains may come from doves and passerine species during the WNV transmission season (36), effective targeting of these preferred hosts with endectocide-treated bird feed could result control of WNV transmission. This use of endectocide-treated feed in wild birds to target WNV transmission could also address some of the concerns of current WNV control methods previously mentioned, as it could be employed by individuals such as farmers and ranchers to achieve localized WNV control around individual properties or neighborhoods.

This study proposes the use of IVM-treated bird feed as a systemic endectocide in wild birds to target *Cx. tarsalis* to disrupt WNV transmission. The following specific aims were developed to address this hypothesis and develop a better understanding of the use of systemic endectocides to control arbovirus transmission.

(I.) Characterize the effects of IVM-treated feed in chickens and wild birds and mosquitoes fed on treated birds (Chapter 2)

(II.) Characterize IVM pharmacokinetics in vertebrates and mosquitoes to better understand the use of IVM as a systemic endectocide (Chapter 3)

(III.) Conduct pilot field trials to test plausibility and efficacy of IVM-treated bird feed to control WNV transmission (Chapter 4)

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CHAPTER 2 CHARACTERIZATION OF IVERMECTIN IN BIRDS AND *CULEX TARSALIS*

2.1 Introduction

While ivermectin (IVM) has been studied as a systemic endectocide for control of ticks and tick-borne pathogens (1, 2) as well as malaria (3–5), it has not been explored as a systemic endectocide to target arbovirus transmission. Given the dynamics of WNV transmission and the current control interventions available, the use of IVM as a systemic endectocide in treated bird feed to target mosquito vectors should be considered as WNV transmission control strategy.

West Nile virus (WNV) is the leading cause of arboviral neuroinvasive disease in the United States (6). Control of WNV transmission remains focused on vector control through larvicide and adulticide applications (7). Larvicidal applications are generally preferred to adulticide applications as they are more cost-effective and less environmentally damaging due to more direct and efficient targeting of mosquitoes (8, 9). While previous studies have demonstrated the effectiveness of larvicide applications to catch basins, a common *Culex* larval habitat, in reducing the number of mosquitoes (10, 11), the efficacy may vary significantly with suboptimal catch basin design or environmental conditions (12, 13). Previous studies have demonstrated that aerial spraying is effective in reducing target mosquito populations (14–17). Further studies have proposed a link between aerial adulticide applications and reductions in human WNV cases in a treated area relative to an untreated area (16) and entomological measures of WNV risk (17). While ground ultra-low volume application of adulticides may reduce target mosquito populations under ideal conditions, studies have provided inconclusive data on their effect on WNV infection rates in mosquitoes or subsequent viral transmission (18–21). Additionally, while optimally calibrating adulticide applications to host-seeking and active

times for target vector species can lessen impact on nuisance species, the potential for off-target effects remains (22–24). Insecticide applications also often face community opposition due to environmental and toxicity/allergenicity concerns (25–29) and are often restricted to urban and semi-urban communities that can afford to fund them (30, 31).

The highest WNV disease incidence occurs along the Great Plains region of the United States (32), as the irrigated agriculture provides a supportive habitat for the main WNV enzootic and bridge vector of the region, *Culex tarsalis* (33). WNV is maintained in an enzootic cycle between *Culex* mosquitoes and avian hosts, therefore, blood meals by *Cx. tarsalis* from preferred avian species may be utilized to selectively target adult females through their blood feeding behavior. Given that the majority of *Cx. tarsalis* blood meals on the northern Colorado plains may come from doves and passerine species during the WNV transmission season (34), effective targeting of these preferred hosts with endectocide-treated bird feed could result control of WNV transmission.

This chapter describes the characterization of IVM in both birds and *Cx. tarsalis*. Effects of IVM on *Cx. tarsalis* survival were examined using both *in vitro* membrane blood meals and direct blood feeding on IVM-treated birds. Chickens and wild Eurasian collared doves fed solely on IVM-treated bird feed concentrations up to 200 mg IVM/kg feed exhibited no signs of toxicity, and most *Cx. tarsalis* that blood fed on these birds died compared to controls. Mosquito survivorship following blood feeding correlated with IVM serum concentrations at the time of blood feeding, which dropped rapidly after the withdrawal of treated feed. These results suggested IVM-treated bird feed should be further explored as a hyper-localized control strategy for WNV transmission.

2.2 Materials and Methods

Mosquito Assays

Cx. tarsalis (Bakersfield colony) were reared in standard insectary conditions (28 ˚C, 16:8 light cycle). Mosquito bioassays for *in vitro* lethal concentrations were performed by adding drug (eprinomectin, selamectin, and IVM) into blood at serial dilutions for artificial membrane feeds. Following feeding, *Cx. tarsalis* were knocked down with CO_2, and fully-engorged females were collected and held for 5 days. For all bioassays, mosquito mortality was recorded every 24 hours and analyzed using Kaplan-Meier survival curves and Mantel-Cox (log-rank) test. LC_{50} values were calculated using nonlinear mixed model with probit analysis.

In vitro membrane blood feeds were also used to test the effects of IVM and WNV on *Cx. tarsalis* mortality. The WNV strain used was a 2012 Colorado isolate propagated in Vero cells, negative controls were DMEM and DMSO at the same volumes as WNV and IVM respectively. For the concurrent feed of WNV and IVM, IVM at 73.66 ng/mL (LC_{75}) and WNV at low titer ($5x10^5$ PFU/mL) or high titer (10^7 PFU/mL) were fed in a membrane blood meal to *Cx. tarsalis* and mortality was observed as described above. For the WNV-exposure followed by an IVM feed, mosquitoes were fed a first blood meal containing 10^7 PFU/mL of WNV. Fully engorged females were sorted and held for 10 days, then fed a second blood meal containing 73.66 ng/mL IVM. Fully bloodfed females were sorted and mortality observed.

Mosquito bioassays following bird blood feeding were conducted on the last day of the IVM feed regimen for each group and for two days following IVM feed removal. For direct feeding on birds, the downy breast feathers were trimmed, and the exposed bird breast was placed on top of the mosquito cage. The birds were gently restrained for 30 minutes while the mosquitoes blood fed through the mosquito cage organdy. For serum replacement feedings,

frozen chicken serum was used in reconstituted blood meals using red blood cells from defibrinated calf blood (35, 36).

Birds

Four to six-week old white leghorn chickens were divided into three groups (n=4) that were housed separately. Chickens were provided clean water daily and either IVM-feed or control (untreated) feed consisting of a cracked corn mix (Chick Start and Grow, Northern Colorado Feeders Supply) for 3 or 7 consecutive days. IVM-treated feed consisted of two formulations: an Ivomec formulation where liquid Ivomec (Merial) was mixed directly into the cracked corn mix and a powder IVM formulation where powder IVM (Sigma-Aldrich) was mixed into all-purpose flour at 5% (w/w) and then added to the cracked corn mixture to aid in even powder distribution. Chickens were fed *ad libitum* and feed consumed by each group was measured daily. Chickens were weighed daily and observed for clinical signs of toxicity, including diarrhea, mydriasis, ptosis, stupor and ataxia. The amount of chicken feed consumed was compared between groups using a t-test and chicken growth rates were compared using linear regression implemented. Blood was collected from these chickens through venipuncture at the end of their IVM feed regimen and for two days following IVM feed withdrawal. Serum was then isolated from the blood samples and stored at -80°C until further analysis.

Eurasian collared doves *(Streptopelia decaocto)* were captured by mist net in Wellington, CO and brought back to CSU. They were housed in groups of three within cages and provided *ad libitum* clean water and either control feed or powder IVM formulation feed at 200 mg IVM/kg of feed for 10 days.

IVM extraction and derivatization

IVM was extracted from serum following methanol precipitation. Four hundred µL of methanol was added to 100 µL serum and vortexed for 1.5 min. Methanol precipitation was carried out at -80 ˚C overnight. Samples were centrifuged for 30 min at 16,000 x g. Supernatants were transferred and evaporated to dryness using a Speedvac concentrator (Savant). The dry residue was dissolved in 15 µL acetonitrile. Samples were derivatized according to previously published literature (37).

HPLC-fluorescence quantification

A Waters 700 autosampler system was used to quantify IVM by high-performance liquid chromatography (HPLC)-fluorescence. A mobile phase of acetonitrile/water (3:1, v/v) was pumped through a C8 column (Waters, XBridge BEH C8 XP, 130 Å, 2.5 µm, 2.1x100 mm) at a rate of 0.45 mL/min. Excitation and emission spectra were 365 and 470 nm, respectively. Ten µL of derivatized sample was injected by the autosampler. Quantified chicken sera concentrations were correlated to cumulative mosquito mortality from blood fed *Cx. tarsalis* using Pearson correlation.

Precision was quantified as coefficient of variation (%CV). This was calculated interday and intraday, evaluating drug-free chicken serum samples (n=5) fortified with IVM at 25, 50, and 100 ng/mL. Instrument CV was 6.11%. Intraday CV ranged between 4.36 and 9.77%. Interday reproducibility was 15.39%. Retention time CV was 1.77%.

The method was linear across the range tested in the calibration curve (3.125 – 100 ng/mL). Linear regression curves containing fortified IVM serum samples with concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 ng/mL had a R-square value of 0.9974.

2.3 Results

Mosquito bioassays following membrane blood meals

Mosquitocidal concentrations of IVM, selamectin, and eprinomectin were determined with mosquito bioassays following blood feeds with serially diluted drug. IVM had the lowest LC_{50} concentration at 49.94 ng/mL (Table 1) as compared to eprinomectin with a LC_{50} of 101.59 ng/mL and selamectin with a LC_{50} of 151.46 ng/mL. Given that it had the lowest effective concentrations among the drugs tested, IVM was chosen for further characterization in mosquitoes and birds.

Table 2.1 Lethal concentrations (LCx) of ivermectin (IVM) for Cx. tarsalis. Mosquitoes were fed serial dilutions of IVM in membrane blood meals. Brackets indicate 95% confidence intervals.

$LC_{(x)}$	[IVM] (ng/mL)
LC_5	19.35 [10.52-26.80]
LC_{10}	23.86 [14.29-31.62]
LC_{25}	33.85 [23.58-42.10]
LC_{50}	49.94 [39.71-59.93]
LC_{75}	73.66 [61.37-92.96]
LC_{100}	104.52 [84.38-149.54]

Potential interactions of IVM and WNV on *Cx. tarsalis* survival were assessed in a simultaneous blood meal of IVM and WNV. WNV (both low and high titer) exposure in the absence of IVM did not affect *Cx. tarsalis* survival over 5 days immediately after the blood meal (Fig. 2.1) or following a second untreated blood meal 10 days later (Fig. 2.2). On the other hand, *Cx. tarsalis* given a concurrent blood meal containing low-titer WNV and IVM exhibited significantly reduced survival compared to the control group not fed WNV ($p=0.0268$) (Figure 2.1A). However, there was no significant difference ($p=0.2529$) in survival between *Cx. tarsalis* fed a concurrent blood meal containing high titer WNV and IVM compared to the control (Figure 2.1B).

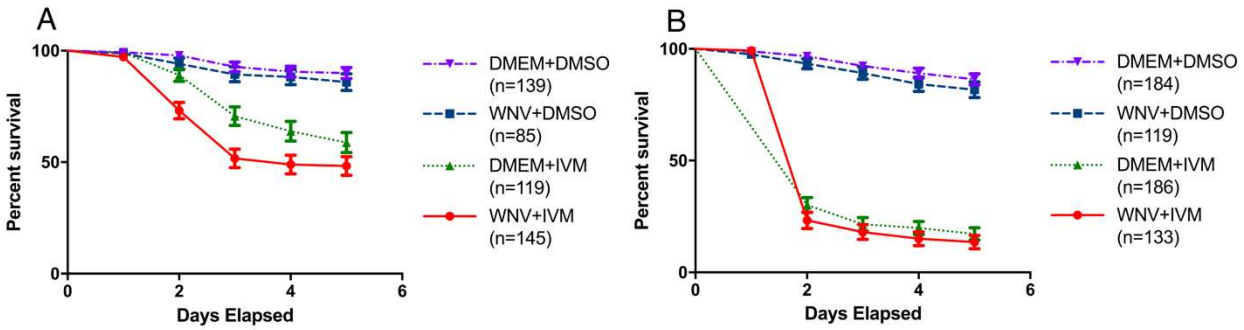

Figure 2.1. *Cx. tarsalis* survivorship following a concurrent blood meal of IVM and WNV. Mosquitoes were fed a membrane blood meal containing IVM at 73.66 ng/mL (LC75) and WNV at a titer of (A) 5x105 PFU/mL or (B) 107 PFU/mL. Error bars indicate standard error of the mean.

Similarly, *Cx. tarsalis* given a first blood meal of either DMEM control or high titer WNV, and then a second blood meal containing IVM 10 days later showed no significant differences in survivorship (p=0.1637) (Figure 2.2).

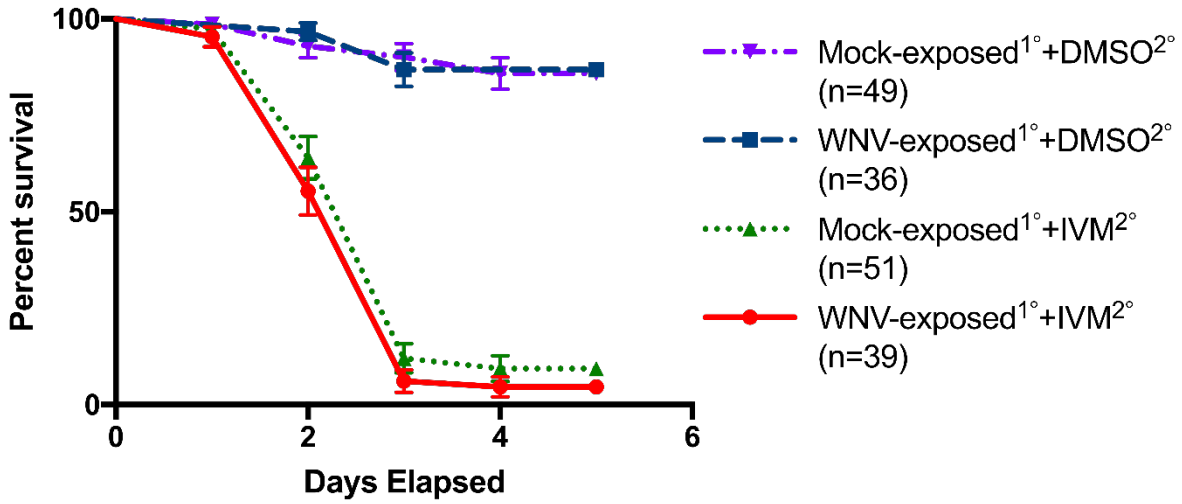

Figure 2.2. *Cx. tarsalis* survivorship following an IVM blood meal after previous WNV exposure. Mosquitoes were fed a second membrane blood meal of IVM at 73.66 ng/mL given 10 days after a first blood meal of 10^7 PFU/mL WNV. Error bars indicate standard error.

Safety and consumption of IVM feed in birds

There were no observable clinical signs of IVM neurotoxicity - diarrhea, mydriasis, ptosis, stupor, and ataxia – in bird groups that consumed either Ivomec or powder formulations of IVM at 200 mg IVM/kg of feed.

For the Ivomec formulation of IVM-treated feed at 200 mg IVM/kg of feed, the chickens consumed an average 59.3 g of feed per chicken daily. This was significantly less than the corresponding control group average daily consumption of 121.6 g per chicken ($p=0.0045$). This difference in consumption between Ivomec and control groups also likely contributed to the significant difference ($p < 0.0001$) in the rate of weight gain between Ivomec and control groups as determined using linear regression ($F=98.64$) (Figure 2.3). For the powder formulation of IVM-treated feed, the IVM group consumed 60.97 g of feed per chicken each day, which was not significantly different from daily control group consumption of 55.2 g of feed per chicken. This was also reflected in similar rates of weight gain between powder IVM and control groups.

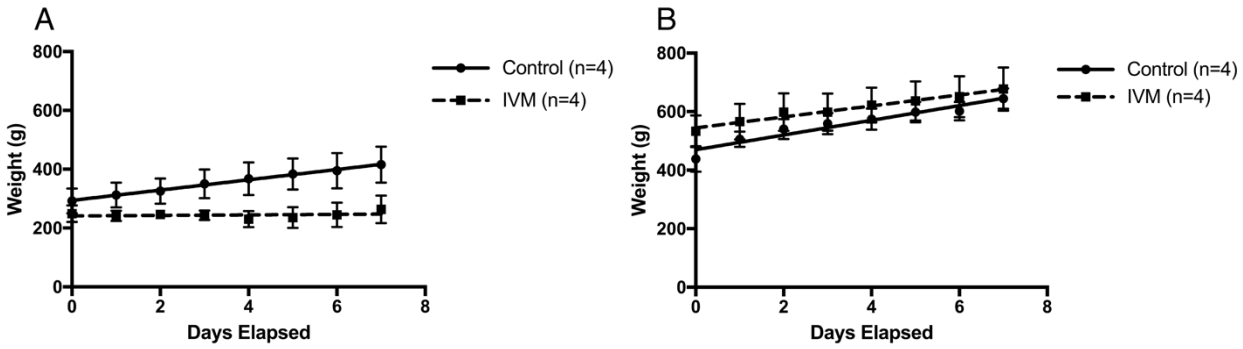

Figure 2.3 Linear relationship between chicken weight and days elapsed. Solid bars indicate control groups and dashed lines indicate ivermectin-treated groups of chickens fed (A) Ivomec-formulation feed or (B) powder IVM-formulation feed. Error bars indicate standard deviation.

Mosquito bioassays from IVM-treated birds

Figure 2.4 represents *Cx. tarsalis* survival following blood feeding on chickens that were fed different concentrations of IVM-treated feed for 7 days. There was no significant difference

between mosquito survivorship following their blood meals taken from IVM-treated chickens and control chickens at 50 mg IVM/kg of feed ($p=0.064$). There was a significant difference in mosquito survivorship at 100 mg IVM/kg of feed ($p<0.001$) with 64.4% survival in mosquitoes blood fed on IVM-treated chickens and 96.8% survivorship in mosquitoes blood fed on control chickens. The largest difference in mosquito survivorship ($p<0.0001$) following blood feeding on control and IVM-treated chickens was at 200 mg IVM/kg of feed with 4.8% survival in mosquitoes blood fed on IVM-treated chickens and 100% survival in mosquitoes blood fed on control chickens. All subsequent experiments used IVM-treated feed at 200 mg IVM/kg of feed.

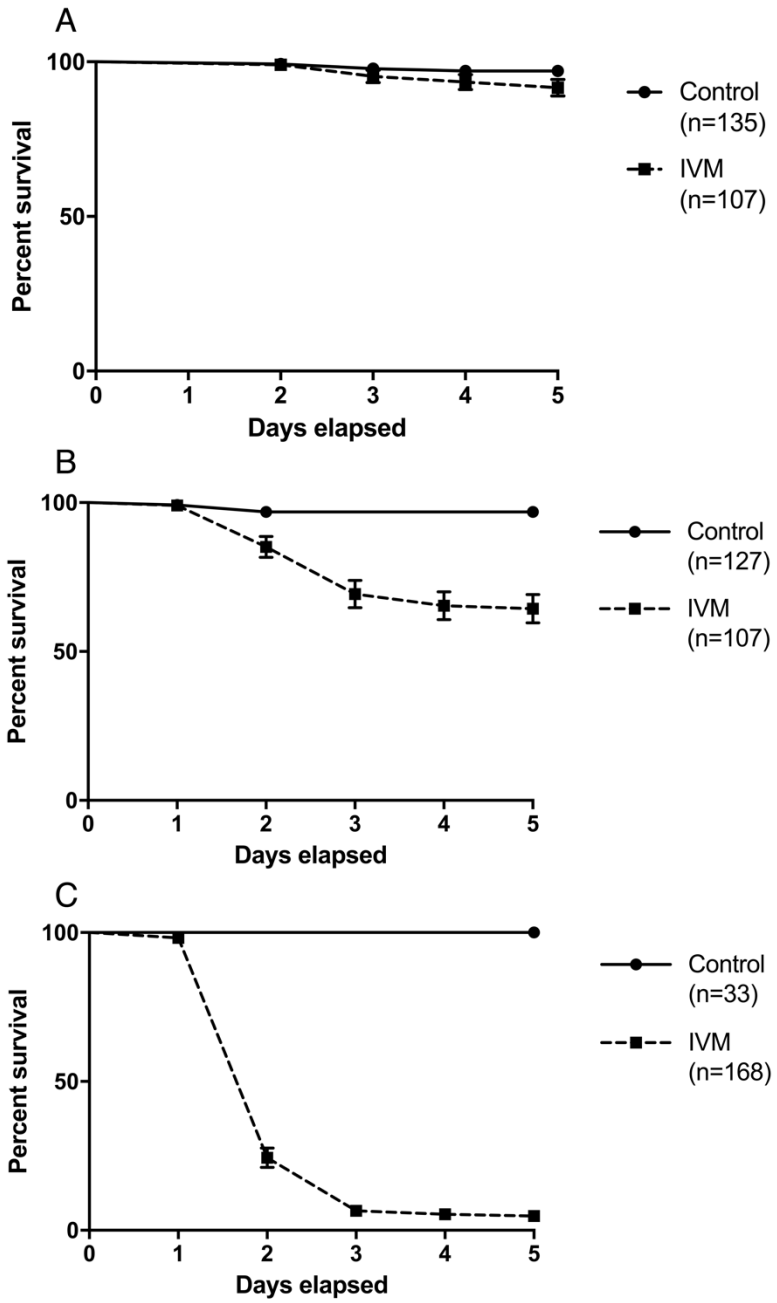

Figure 2.4 *Cx. tarsalis* survivorship following direct blood feeding on Ivomec-treated chickens. Mosquito survivorship is shown following direct blood feeding on chickens that were fed Ivomec-formulation feed for 7 consecutive days at concentrations of (A) 50 mg IVM/kg of feed, (B) 100 mg IVM/kg of feed, and (C) 200 mg IVM/kg of feed. Error bars indicate standard error.

For the Ivomec formulation at 200 mg IVM/kg of feed, there was a significant difference in survival between mosquitoes fed on control chickens and mosquitoes blood fed on chickens consuming Ivomec feed for either 3 or 7 days ($p<0.0001$) (Figure 2.5). At 3 days of Ivomec feed,

there was 60.5% survival in *Cx. tarsalis* blood fed on control chickens as compared to 10.8% survival in *Cx. tarsalis* blood fed on chickens consuming Ivomec feed. Mosquito survivorship resulting from blood feeding on chickens given Ivomec feed for 7 days was 23.9%, while mosquitoes blood fed on control chickens had 81.3% survivorship. This mosquitocidal effect of IVM was not long-lived once Ivomec feed was withdrawn for both 3 and 7 day Ivomec groups, as the difference between Ivomec and control mosquito survival curves decreased. At 1 day post-Ivomec withdrawal following 3 days of Ivomec feed, mosquito survivorship resulting from blood feeds on Ivomec-fed chickens was 61.7% as compared to 74.2% mosquito survivorship from blood feeding on control chickens ($p=0.0830$). At 1 day post-Ivomec removal following 7 days of Ivomec feed, there was still a significant difference in mosquitoes blood fed on chickens given Ivomec feed with 53.4% survival as compared to 78.5% survival from mosquitoes blood fed on control chickens. Following 2 days of IVM withdrawal, the mosquitocidal effect of IVM is essentially depleted for both 3 and 7 day Ivomec groups and there is no significant difference between survivorship of mosquitoes blood fed on either 3 or 7 day Ivomec chickens and their respective controls. In addition, there was no observable difference in mosquito survival curves following direct blood feeding on chickens given either 3 days or 7 days of IVOMEC-treated feed. Furthermore, the mosquito survival following blood feeding on chickens after IVM withdrawals for 1 and 2 days did not differ between the 3 and 7 day IVM feed regimens.

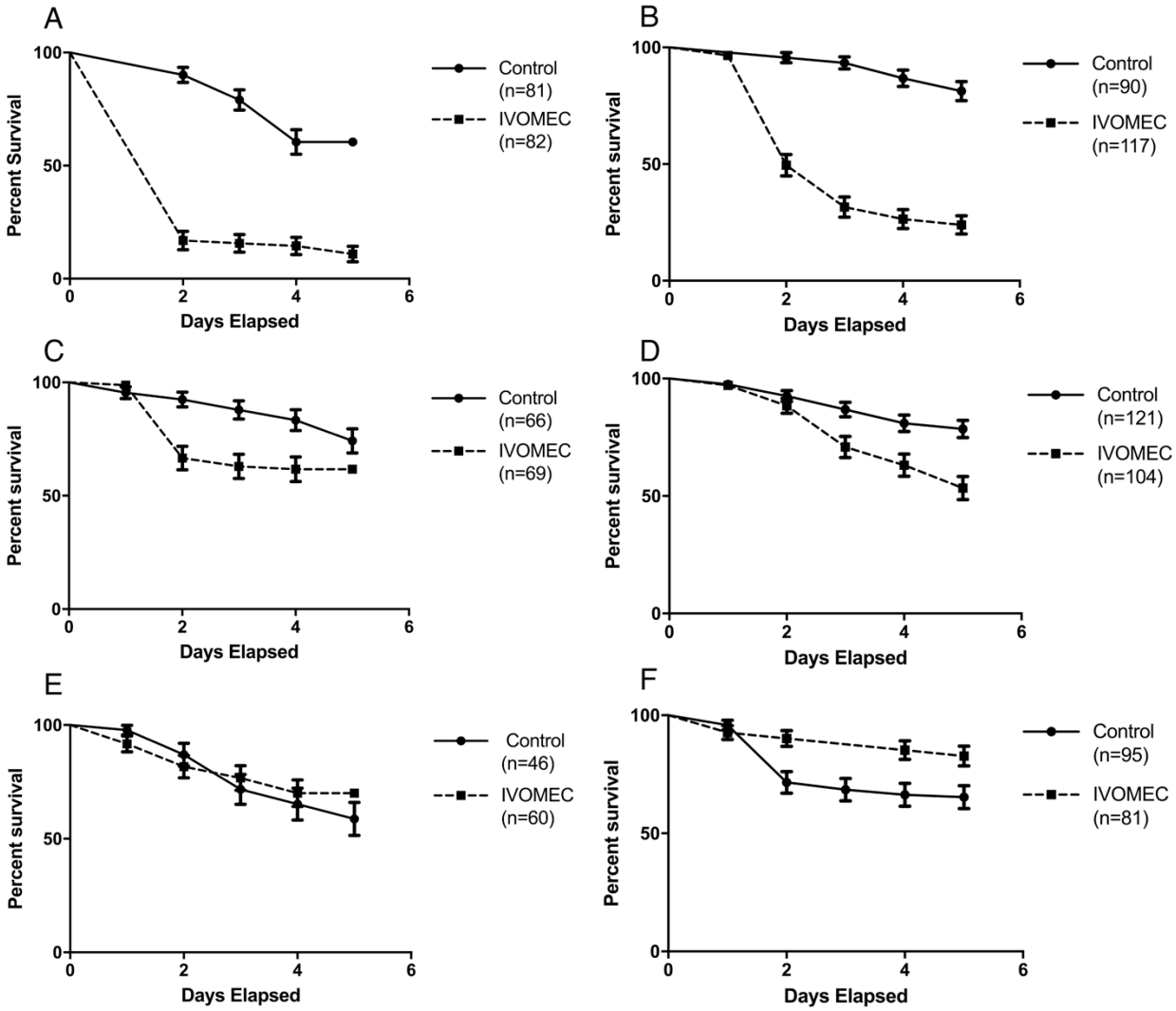

Figure 2.5 *Cx. tarsalis* survivorship following direct blood feeding on chickens fed Ivomec feed for 3 and 7 days. Mosquito survivorship is shown following direct blood feeding on chickens given Ivomec-formulation feed at a concentration of 200 mg IVM/kg of feed for (left panels: A, C, E) 3 and (right panels: B, D, F) 7 days. (Top panels: A, B) Blood feeding occurred on the last day treated feed was given to the IVM groups. (Middle panels: C, D) Blood feeding occurred one day after treated feed was withdrawn from the IVM groups. (Bottom panels: E, F) Blood feeding occurred on the second day after treated feed was withdrawn from the IVM groups. Error bars indicate standard error.

For chickens fed the powder formulation of IVM at 200 mg IVM/kg of feed, there was also significantly decreased survival observed in mosquitoes blood fed on chickens given this IVM formulation as compared to control chickens (Figure 2.6). Data from the Ivomec formulation and preliminary powder formulation bioassays indicated no differences between

mosquitocidal effects for groups given IVM for 3 or 7 days, so subsequent experiments focused on the 7 day time point. A direct feed of mosquitoes blood fed on chickens given powder IVM feed for 7 days had 7.7% survivorship as compared to 74.3% survivorship from mosquitoes blood fed on control chickens. A serum-replacement blood feed using serum from chickens given powder IVM for 7 days resulted in 21.0% mosquito survival while mosquitoes blood fed on serum from control chickens had 83.3% survival. At 1 day post-IVM removal following 7 days of powder IVM feed, there was still a significant difference between mosquitoes directly blood fed on IVM-fed chickens with 9.1% survivorship compared to 100% survivorship in mosquitoes directly blood fed on control chickens. However, this mosquitocidal effect was not apparent in a serum-replacement feed using blood from 1 day post-IVM feed removal. At 2 days post-IVM feed removal, blood from treated chickens was no longer mosquitocidal in either blood feeding assay.

There were significant differences in survival curves for mosquitoes blood fed directly on chickens as compared to serum-replacement feeds using sera drawn from these corresponding chickens (Figure 2.6). For direct blood feeds and serum-replacement feeds from chickens given 7 days of IVM feed, mosquitoes blood fed directly on chickens had 72.5% survival on day 5 post-blood feed (PBF), while mosquitoes blood fed on sera from these chickens had 21.0% survival on day 5 PBF. The survival curves were significantly different by the log-rank test ($p<0.0001$), and the hazard ratio (logrank) of direct feed survival to serum-replacement feed survival was 2.007. There was also a significant difference between direct and serum-replacement feed mosquito survival curves from chickens 1 day post-IVM withdrawal following 7 days of IVM feed ($p<0.0001$). Mosquito survivorship from a direct feed from chickens 1 day post-IVM was 9.1% at day 5 PBF, while serum-replacement feeds using sera from the corresponding chickens

resulted in 18.1% survival at day 5 PBF. The hazard ratio (log-rank) of direct feed survival to serum-replacement survival from mosquitoes fed on chickens at this time point was 6.742.

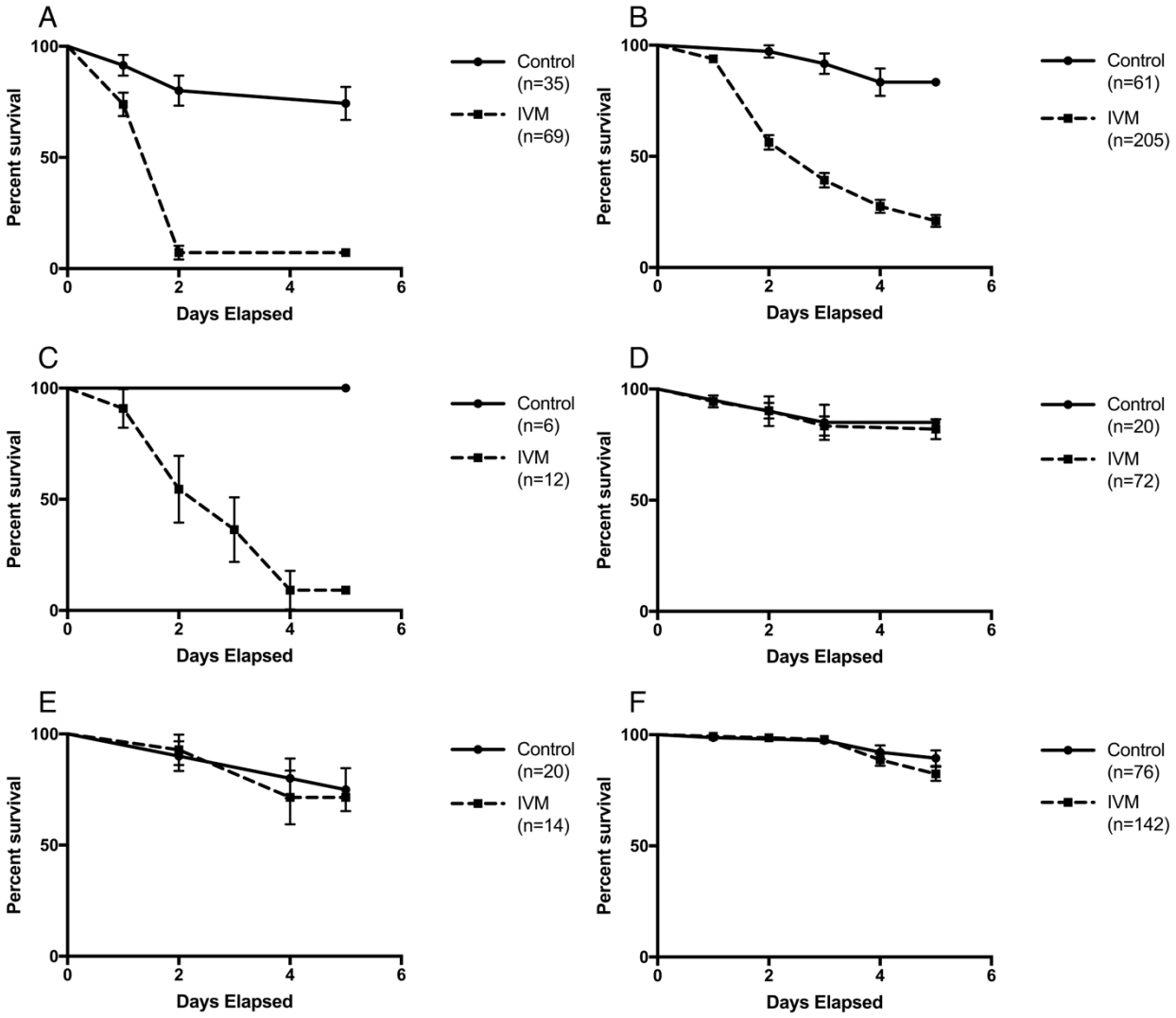

Figure 2.6 *Cx. tarsalis* survivorship following direct (left panels: A, C, E) or serum-replacement (right panels: B, D, F) blood feeding on chickens given powder IVM-feed at a concentration of 200 mg IVM/kg of feed for 7 days. (Top panels: A, B) Blood feeding occurred on, or using serum taken on, the last day treated feed was given to the IVM groups. (Middle panels: C, D) Blood feeding occurred on, or using serum taken on, the day after treated feed was withdrawn from the IVM groups. (Bottom panels: E, F) Blood feeding occurred on, or using serum taken on, the second day after treated feed was withdrawn from the IVM groups. Error bars indicate standard error.

Direct blood feeds of *Cx. tarsalis* were also conducted on wild caught *S. decaocto* fed a powder IVM formulation of feed at 200 mg IVM/kg of feed in the lab (Figure 2.7). There was a

significant difference ($p<0.0001$) with 11.5% survivorship in *Cx. tarsalis* blood fed on IVM-treated doves as compared to 85.7% survivorship from mosquitoes blood fed on control doves. Additionally, there were no clinical signs of IVM toxicity observed in this treated bird species.

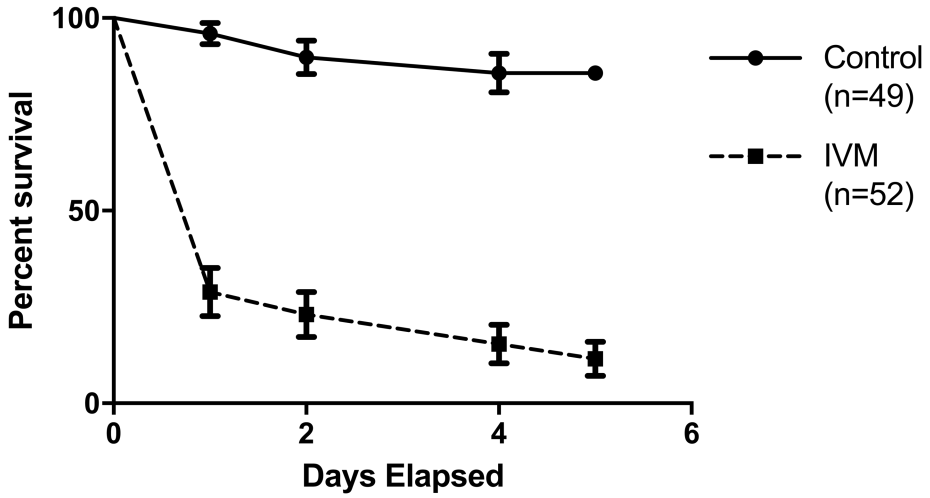

Figure 2.7 *Cx. tarsalis* survivorship following feed IVM-fed Eurasian Collared Doves. Mosquito survivorship is shown following direct blood feeding on captured wild Eurasian Collared Doves fed powder IVM-feed at a concentration of 200 mg IVM/kg feed for 7 to 10 days. Error bars indicate standard error.

IVM serum concentrations

There were no significant differences in the average IVM serum concentrations among the chicken groups on their last day of IVM-feed consumption (Figure 2.8). The average IVM serum concentrations (with SD) were 88.575 (±43.613) ng/mL for 3 day Ivomec, 45.255 (±70.051) ng/mL for 3 day powder IVM, 21.910 (±20.914) ng/mL for 7 day Ivomec, 45.745 (±33.852) ng/mL for 7 day powder IVM. There was no significant difference in IVM serum concentrations among the groups regardless of IVM feed formulation or time for which the IVM feed was consumed.

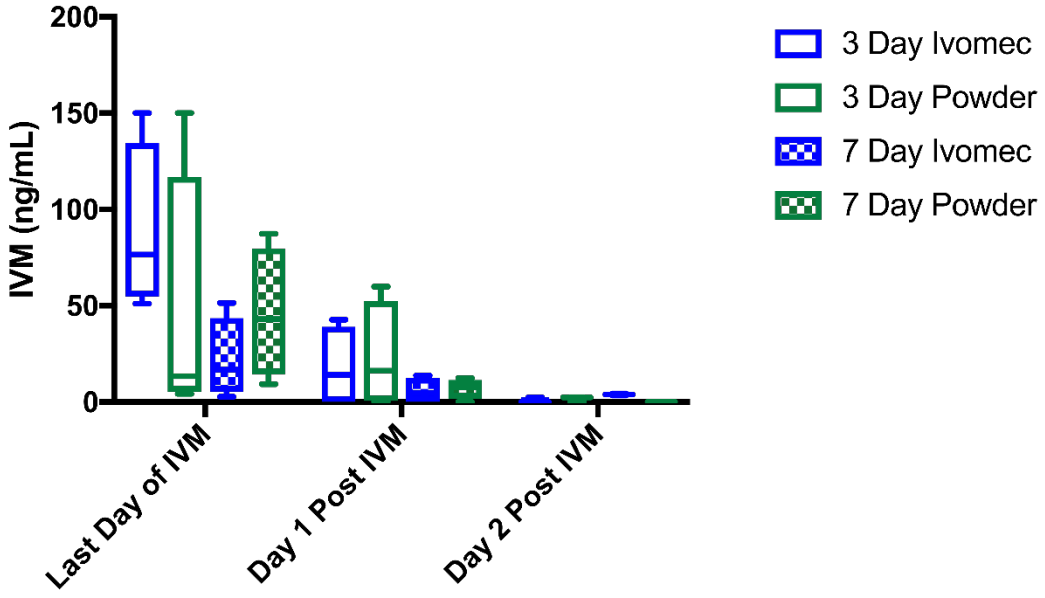

Figure 2.8 Average chicken sera concentrations over time. Chicken sera IVM concentrations measured in IVM-treated groups and taken on the last day treated feed was given, or one or two days after treated feed was withdrawn. Lines indicate median values, boxes indicate 25-75 percentiles, whiskers indicate minimum and maximum values.

IVM serum concentrations were correlated to resulting mosquito mortality from feeding on these corresponding IVM-treated chickens (Figure 2.9). There was higher correlation between IVM serum concentrations and mortality from serum-replacement feeds with a Pearson r of 0.8098, while the correlation between IVM serum concentrations and mortality from direct feeds was 0.3273.

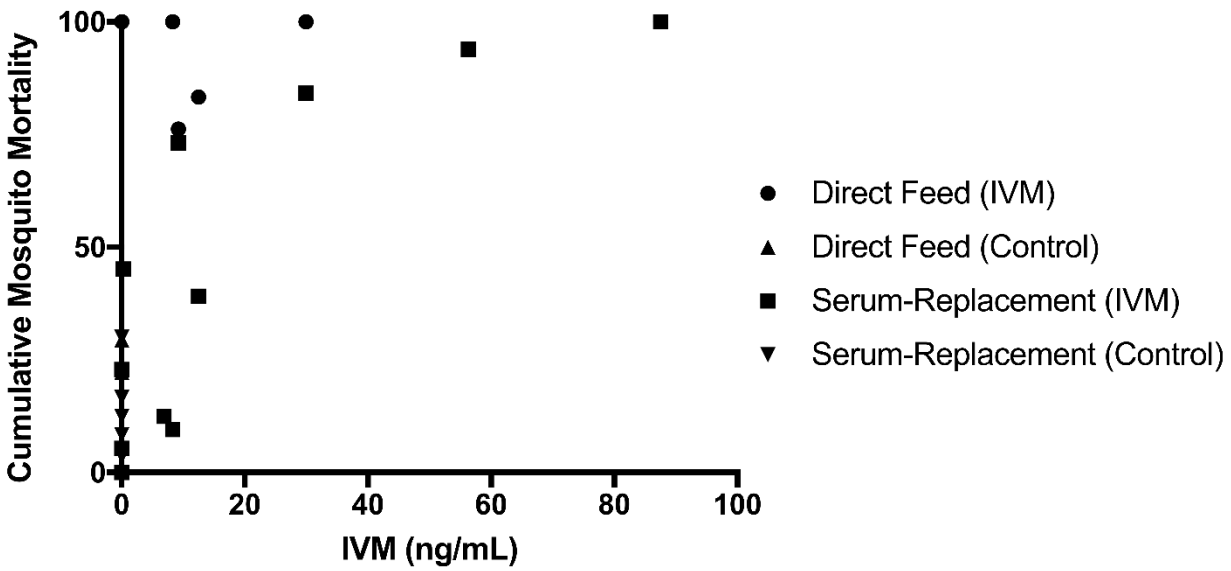

Figure 2.9 Individual chicken serum concentrations versus cumulative mosquito mortality. Individual chicken serum IVM serum concentrations versus corresponding cumulative *Cx. tarsalis* mortality on day 5 post blood feeding for both direct and serum-replacement blood feeds on chickens given powder IVM-formulation feed at a concentration of 200 mg IVM/kg of feed.

2.4 Discussion

IVM was determined to be the most effective endectocide tested with the lowest lethal concentrations for *Cx. tarsalis.* In addition, there did not appear to be a synergistic effect of IVM and WNV on *Cx. tarsalis* mortality in either simultaneous feeds or feeds with previous exposure to WNV followed by IVM consumption. There was a statistical difference between survival curves of *Cx. tarsalis* fed a concurrent blood meal of a low WNV titer IVM compared to *Cx. tarsalis* fed only IVM. However, this decreased survivorship was likely due to the variable survival response of mosquitoes to IVM, rather than a biologically significant interaction between WNV and IVM as there was no survival difference between mosquitoes fed a concurrent higher titer WNV+IVM blood meal compared to mosquitoes fed DMEM+IVM. There was also no difference between mosquitoes previously exposed to WNV and then fed IVM as compared to mosquitoes unexposed to WNV and then fed IVM. While there is a study suggesting that IVM can inhibit WNV replication by targeting NS3 helicase activity, this was an

in vitro cell-culture study using mammalian cells, and the concentration of IVM needed to inhibit 50% of the RNA synthesis in the Vero cells infected with WNV was considerably higher than what was achieved in our chickens following IVM feed consumption (38).

No clinical signs of toxicity were observed in any of the chickens for either formulations of IVM feed. This was not surprising as IVM is given therapeutically in bird species in a wide range of doses (0.2 mg/kg to 2 mg/kg), depending on route of administration (39–41). In addition, the mean IVM serum levels observed in chickens ranged from 21.91 ng/mL to 88.58 ng/mL, which is significantly less than the 739.6 (±50.2) ng/mL plasma concentrations observed following subcutaneous administration of IVM in laying hens where there were also no toxic side effects observed (42). Furthermore, Moreno et al. observed detectable levels of IVM in plasma for 7 days following subcutaneous IVM administration without any ill effects, which is longer than our 1-2 days of detectable IVM serum levels following IVM-feed withdrawal (42). However, more detailed studies of IVM toxicity should be conducted, as there have been neurotoxic effects identified in pigeons following long-term consumption of avermectin, of which IVM is a safer derivative. Chen et al. observed clinical signs of neurotoxicity ranging from the more mild, reduced activity and food intake, to the more severe, ataxia and spasms, following avermectin consumption for 60 days at 20 mg/kg or for 30 days at the higher dose of 60 mg/kg (43).

Chickens fed the powder IVM formulation and their respective controls consumed similar amounts of food. However, chickens fed the Ivomec formulation consumed significantly less feed compared to their corresponding controls. This is may be a result of the glycerol formal and propylene glycol carriers that could give an unpleasant taste, as propylene glycol has been identified as a unpleasant and unpalatable feed additive in cattle (44). The decreased Ivomec feed

consumption relative to control feed consumption was likely responsible for the significantly reduced rate of weight gain in the Ivomec group as compared to controls.

Chickens that consumed either powder or Ivomec formulations at 200 mg IVM/kg of feed developed mosquitocidal concentrations of IVM in their blood within 3 days, as demonstrated by both the IVM serum concentrations of the chickens as well as the significant difference in survival curves of mosquitoes blood fed on IVM-fed chickens as compared to mosquitoes blood fed on control chickens. There were no notable differences between either IVM formulations in mosquitocidal efficacy, for either time to achieve a mosquitocidal effect or its persistence in chicken serum following IVM withdrawal. Furthermore, there were no significant differences between 3 and 7 days of IVM consumption, as 7 days of IVM consumption did not result in greater mosquito mortality, higher IVM serum concentration, or slower elimination of IVM. This is corroborated by the similar IVM serum concentrations at all time points among the different IVM administration times and formulations. Lack of observable toxicity as well as a mosquitocidal effect following consumption of IVM at a concentration of 200 mg IVM/kg of feed was also demonstrated in wild-caught Eurasian Collared Doves, indicating similar efficacy of the approach across bird species and applicability to field situations.

The mosquitocidal effect of IVM in IVM-fed chickens did not extend past one day of IVM-feed withdrawal and this corresponded with the IVM serum concentrations that were generally below detectable limits by two days post-IVM withdrawal. This could potentially be a concern for applying this strategy of IVM-treated bird feed in the field as it would necessitate frequent bird visits to maintain mosquitocidal concentrations of IVM within birds. However, these experiments were conducted in an artificial lab environment using chickens as a model for

wild-caught birds and where colony *Cx. tarsalis* are held in ideal rearing conditions. It is likely that the lethal and sub-lethal effects of IVM would be exacerbated, and therefore longer-lasting, in harsher field conditions as seen with the malaria vector *Anopheles gambiae* when blood fed on IVM in field settings compared to IVM blood feeding tests with the same species in the lab (3). Further studies should include exploring IVM pharmacokinetics in wild bird species and field trials of IVM bird feed should include frequent sampling of wild bird sera for the presence of IVM.

Survivorship in control groups had a greater variation for direct blood feeds (17.75% CV) relative to serum-replacement feeds (3.57% CV), indicating that direct blood feeds may have more inherent variability in mosquito survival. This increased variability in direct blood feeds could be a result of increased mosquito handling and rougher conditions during blood feeding on chickens. It is likely that the smaller sample sizes achieved for direct blood feeds due to the low success of mosquitoes imbibing full blood meals from live chickens also contributed to this higher variability. Similarly, this higher variability in direct blood feed survival could be one explanation for the better correlation between IVM serum concentrations and mosquito survival from serum-replacement feeds as compared to mosquito survival from direct blood feeds.

Another explanation for the lower mosquito survivorship observed from direct blood feeds as compared to serum-replacement feeds is that the IVM within serum extracted from venous blood is not an accurate representation of the IVM concentration in blood from subdermal capillaries on which mosquitoes blood feed. It has been proposed that because IVM is extremely lipophilic and sequestered in fatty tissues, there may exist a concentration gradient of higher IVM in adipose tissue and surrounding capillaries with lower IVM concentrations in venous blood, and therefore, the subdermal capillaries on which mosquitoes blood feed may

present a different IVM concentration as compared to venous blood (45). This is also one explanation for the observation that the IVM serum concentrations in chickens correlated with higher cumulative mosquito mortality than would be predicted from the *in vitro* LC_x values previously calculated. This will be further explored in the next chapter analyzing IVM in mosquito blood meals.

This chapter characterizes IVM as a systemic endectocide in birds and demonstrates that it could potentially be a novel tool to control WNV transmission given that effective mosquitocidal concentrations of IVM can be achieved in birds consuming IVM-treated feed. Importantly, the birds seemed willing to consume IVM-treated feed and did not display any observable clinical signs for IVM toxicity following consumption, and mosquitoes also willingly fed on IVM-treated birds and died as a result. Further directions could include a more nuanced characterization of any IVM toxicity in multiple bird species and controlled field trials of IVM-treated feed during WNV season to see if there is any resulting reduction in entomological measurements of WNV disease transmission.

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CHAPTER 3 DETECTION AND QUANTIFICATION OF IVM IN BLOOD FED MOSQUITOES

3.1 Introduction

Chapter 2 presented the idea of IVM as a control tool for WNV due to its effect on *Culex* mosquitoes when they bite IVM-treated birds, and IVM has been explored as an integrated control tool for malaria due to its effect on *Anopheles* vectors when they bite IVM-treated people and livestock. However, IVM detection and quantification in these mosquito species has not been published. This is important as IVM pharmacokinetics is generally studied using venous-drawn serum or plasma. These may not be a true representation of the amount of IVM ingested by mosquitoes, as they feed on subdermal capillaries in close proximity to subdermal fat deposits that may have higher concentrations of IVM, given its lipophilic nature. While a study demonstrated that venous and capillary blood show a 1:1 correlation for IVM concentration (unpublished data – ASTMH 2017), this was not determined by directly sampling and testing blood ingested by mosquitoes. For *Anopheles*, there have been documented discrepancies between lethal concentrations as predicted by using known concentrations of IVM in blood for membrane feeding assays when compared to membrane feeding assays conducted using blood from IVM-treated volunteers (1). The authors suggested this could be due to an uncharacterized metabolite or blood meal size differences between membrane and direct feeding assays (1), and perhaps the nature of this discrepancy could be answered with IVM quantification from mosquito blood meals. For *Cx. tarsalis,* lethal concentrations of IVM are lower in direct feeds from chickens compared to those predicted using *in vitro* membrane feeds with known concentrations of IVM, and direct feeds from chickens also had higher mosquitocidal efficacy as compared to serum-replacement feeds using venous-drawn serum (Chapter 2). Quantifying IVM

concentrations in individual *Cx. tarsalis* blood meals between these methods may explain the mosquitocidal differences between these assays.

Integrated malaria- and WNV-control methods using IVM also rely on adequate coverage of IVM within mosquito populations, and therefore, a method to detect and quantify IVM within mosquito blood meals is important. Critical questions within modelling IVM as a control strategy for malaria include adequate dosing and timing of IVM administration, and sufficient coverage within the human population (2–5). An ability to quantify IVM in *Anopheles* blood meals is an important step towards addressing these questions. In addition, sub-lethal effects of IVM can have significant effects on vector behavior, fecundity, and parasite transmission (4, 6, 7), and quantification of IVM in mosquito blood meals can help define the duration of these sub-lethal effects. Finally, because human WNV disease endpoints are difficult to correlate with vector control interventions (8, 9), assessing IVM coverage within *Cx. tarsalis* blood meals could be a critical link between efficacy of IVM as a systemic endectocide in wild birds and reduced WNV transmission or disease in humans or sentinel animals.

In this study, a HPLC-fluorescence method was developed to detect and quantify IVM in individual blood meals of *An. gambiae* and *Cx. tarsalis.* Variation in individual blood meals for *Cx. tarsalis* and *An. gambiae* taken from both artificial membrane feeders and *in vivo* direct feeds were assessed. The quantity of IVM ingested in individual mosquitoes was also compared to the venous serum concentrations of live animals.

3.2 Material and Methods

Mosquito Feeding

An. gambiae (G3 strain) and *Cx. tarsalis* (Bakersfield strain) were raised in standard insectary conditions (28˚ C, 16:8 light cycle). *In vitro* feeds were conducted by adding IVM to

defibrinated calf blood (Colorado Serum Company), and the blood meal was housed in a glass feeder with a hog gut membrane and warmed to 37˚ C with a circulating water bath. *Cx. tarsalis* were also fed on live chickens dosed with IVM, as described in Chapter 2. Serum-replacement feeds were also conducted as described in Chapter 2. The grackle serum used in the serum-replacement feed was taken from wild grackle caught near an IVM-treated feeder.

IVM Analysis

Individual blood fed mosquitoes were homogenized in 100 µL methanol. 300 µL methanol was added for protein precipitation and samples were precipitated overnight. Samples were centrifuged at 16,000 x g for 30 minutes and 350 µl of supernatant was transferred to a new 1.5 mL tube for evaporation. The dried residue was resuspended in 10 µL acetonitrile (ACN). Derivatization was performed using 10 µL of *N*-methylamidizole:ACN (1:1 v/v), 5 µL triethylamine,15 µL of tri-fluoracetic acetic anhydride:ACN (1:2, v/v), and 5 µL trifluoracetic acid.

A Waters 700 autosampler system was used to quantify IVM by high-performance liquid chromatography (HPLC)-fluorescence. A mobile phase of acetonitrile/water (3:1, v/v) was pumped through a C8 column (Waters, XBridge BEH C8 XP, 130 Å, 2.5 um, 2.1x100 mm) with a guard column of the same chemistry at a rate of 0.45 ml/min. Excitation and emission spectra were 365 and 470 nm, respectively. 12 µL of derivatized sample was injected by the autosampler.

Standard curve for IVM quantification

The standard curve for IVM quantification was generated using *Cx. tarsalis* frozen 0 hours and 12 hours post-blood feed (PBF) and *An. gambiae* frozen 12 hours PBF. Mosquitoes were blood fed serial dilutions of IVM ranging from 0.78 to 100 ng/mL in artificial membrane feeders.

Following blood feeding, engorged females were held in colony conditions until frozen at their respective time point and processed for IVM analysis. 12 µL of derivatized sample were analyzed for *Cx. tarsalis* standard curve and 15 µL were analyzed for the *An. gambiae* standard curve.

3.3 Results

Figure 3.1 depicts HPLC-fluorescence chromatograms from mosquito blood meal substrates and chromatograms from the corresponding individual blood fed mosquitoes. Derivatized IVM corresponds to the peak with an approximate retention time at 8 minutes (black arrow). The area of the peak, or the area under the curve (AUC), is a function of the amount of signal, from IVM in this particular case.

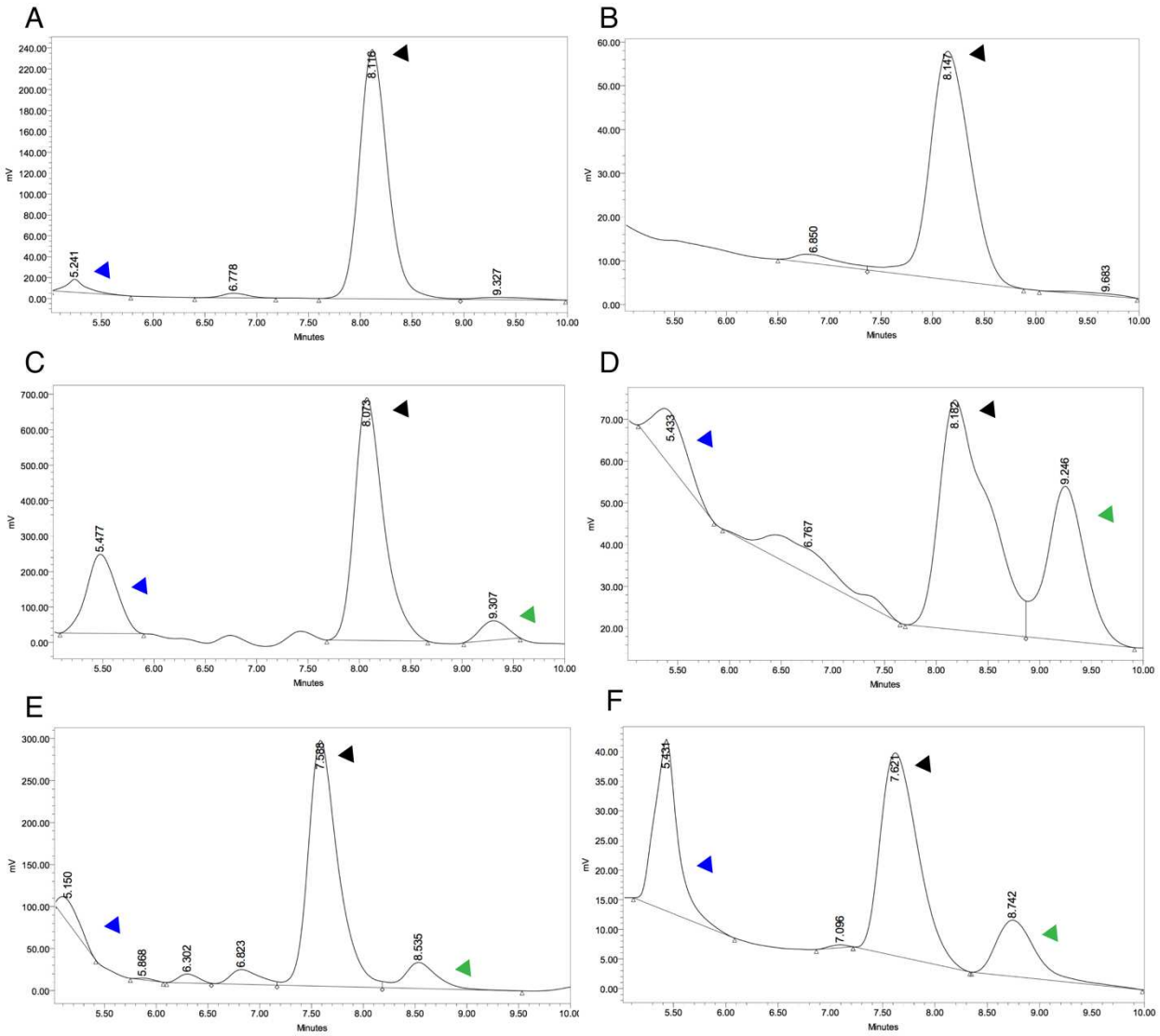

Figure 3.1 Representative chromatograms of HPLC-fluorescence analyses of individual *Cx. tarsalis* mosquitoes fed blood meals and their respective blood meal substrates. Panel A is an analysis of serum from a defibrinated-calf blood meal fortified with 6.25 ng/mL IVM, and panel B is an analysis of an individual *Cx. tarsalis* mosquito that blood fed on the 6.25 ng/mL IVM-fortified calf blood. Panel C is an analysis of serum from a chicken fed 200 mg IVM/kg of feed for 3 days, and panel D is an analysis of an individual *Cx. tarsalis* mosquito that directly blood fed on this chicken immediately preceding the blood collection. Panel E is an analysis of serum from a different chicken fed 200 mg IVM/kg of feed for 3 days, and panel F is an analysis of an individual *Cx. tarsalis* mosquito that fed on a serum-replacement meal using the serum harvested from this chicken immediately preceding the blood collection. Black arrowheads indicate the peak for derivatized IVM. Blue arrowheads indicate a non-specific peak also present in drug-free serum, while green arrowheads indicate a likely IVM metabolite. All mosquitoes were harvested immediately (0 hours PBF) after they ingested their blood meals.

Figure 3.2 shows that the variation between single mosquitoes sampled following direct blood feeds on chickens consuming IVM-treated feed or following IVM-fortified artificial membrane meals are similar. The coefficient of variation (CV) for individual *Cx. tarsalis* fed on chickens dosed with IVM ranged from 0.87% to 67.58%, and the CV for individual *Cx. tarsalis* fed on the artificial membrane feeder was 45.37%. This indicates that variation between individual blood meals was likely not a result of the source.

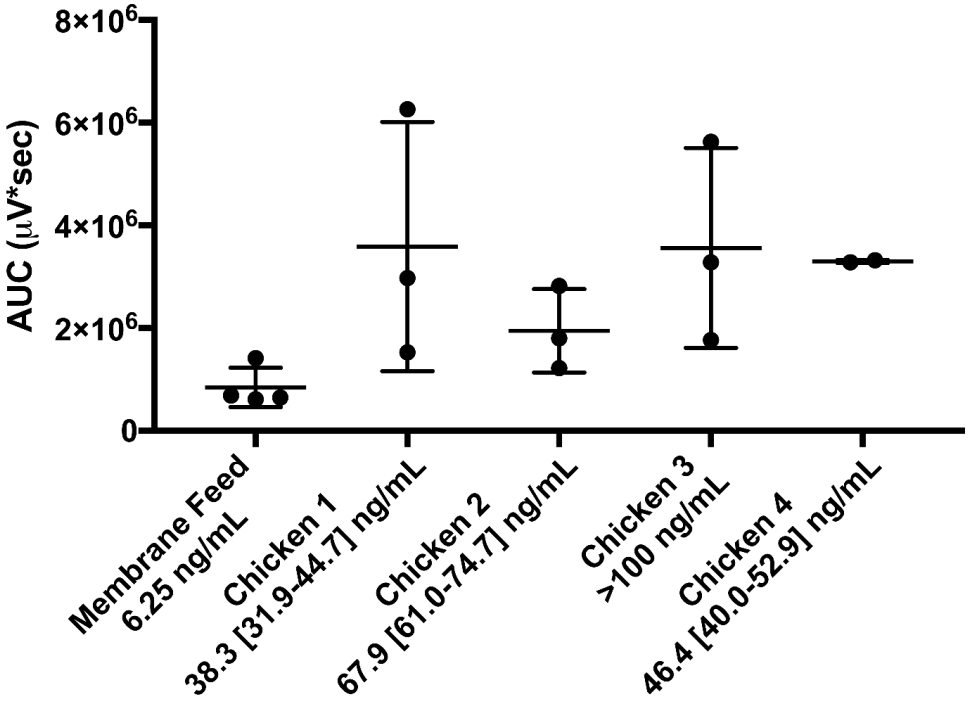

Figure 3.2 Variation for individual blood fed *Cx. tarsalis.* Variation is shown for blood meals taken from an artificial membrane feed of 6.25 ng/mL, and directly from chickens fed 200 mg IVM/kg of feed.

Figure 3.3 represents standard curves of AUC vs. IVM concentration from individual *Cx. tarsalis* mosquitoes blood fed at different concentrations of IVM at both 0 hours and 12 hours PBF. The standard curves were linear from blood meals between 1.56 ng/mL to 100 ng/mL. The R-square values for the standard curves at 0 hours PBF and 12 hours PBF were 0.9798 and

0.9608, respectively. In addition, there was no significant difference between the slopes (p=0.1817) and intercepts (p=0.1914) of the standard curve from mosquitoes sampled at 0 hours or 12 hours PBF.

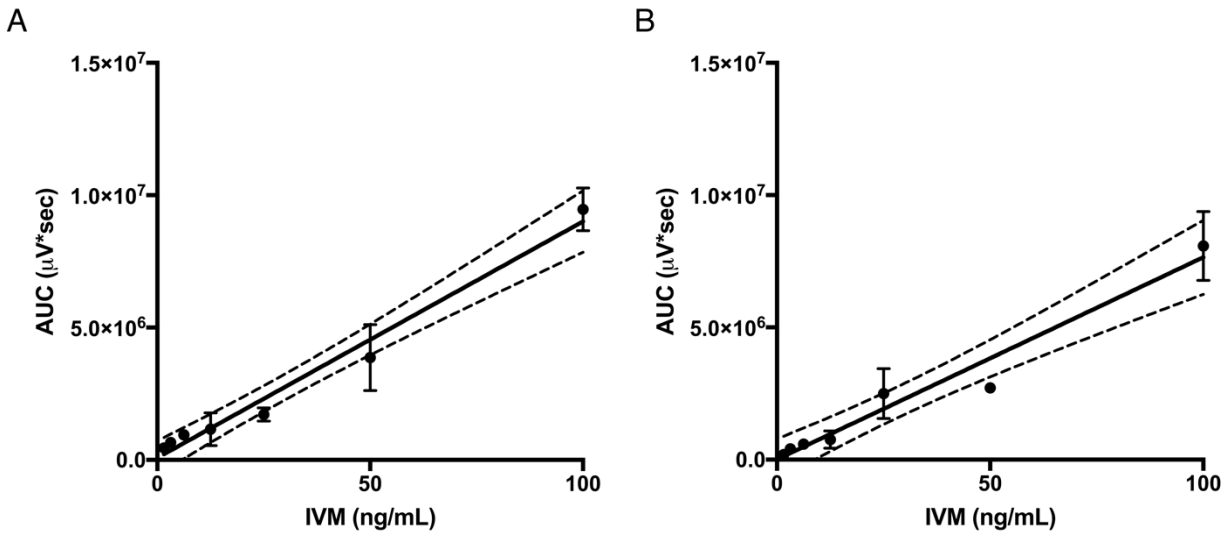

Figure 3.3 Standard curves of AUC vs. IVM concentration from individual blood fed *Cx. tarsalis* mosquitoes. Standard curves were generated using individual blood fed *Cx. tarsalis* (n=3) which ingested IVM-fortified blood in serial dilutions from artificial membrane feeders, ranging from 1.56 ng/mL to 100 ng/mL. Panel A represents mosquitoes frozen at 0 hours PBF and panel B represents mosquitoes frozen 12 hours PBF.

Figure 3.4 demonstrates quantification of individual *Cx. tarsalis* mosquitoes based on the standard curve at 0 hours presented in Figure 3.3. The quantification of IVM in a blood fed mosquito was considered accurate if the 95% confidence interval (CI) for the quantified IVM in the blood fed mosquito overlapped with the 95% CI for the amount of IVM quantified in the bird serum. IVM quantification was accurate for 83% (5/6) of *Cx. tarsalis* directly blood fed on an IVM-treated chicken. However, only one *Cx. tarsalis* from the grackle serum-replacement feed had the expected IVM concentration within its blood meal based on the grackle IVM serum concentration.

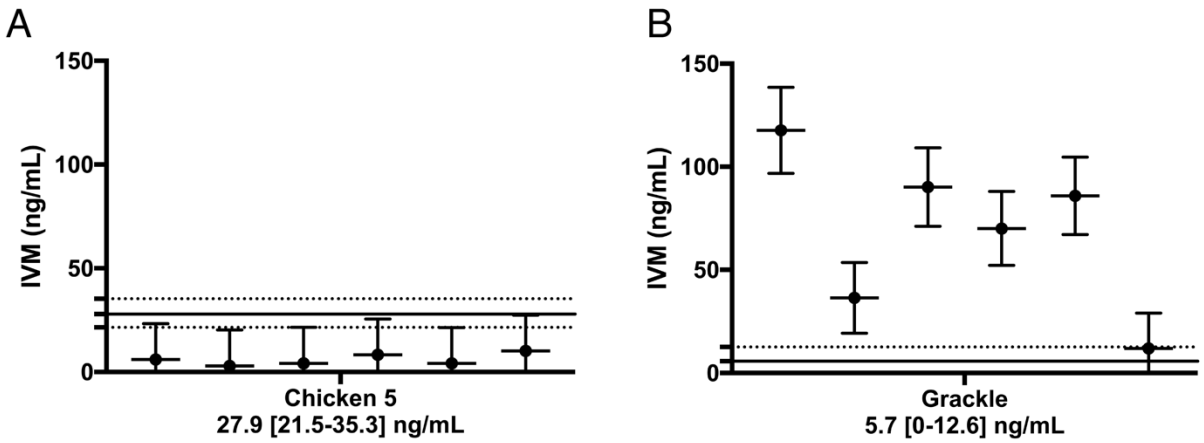

Figure 3.4 IVM quantification in individual blood fed *Cx. tarsalis* mosquitoes. Quantifications were made using the standard curve presented in Figure 3.3 *Cx. tarsalis* were directly fed on Chicken 5 (Panel A), or serum-replacement fed using wild grackle serum (see Chapter 4) (Panel B), and IVM concentrations were determined. Error bars on *Cx. tarsalis* data points indicate 95% CI based on the standard curve. The solid line indicates the quantified bird serum concentration and dotted lines indicate 95% CI based on the standard curve.

Figure 3.5 demonstrates that both the AUC values and their variation from *Culex* and *Anopheles* mosquitoes blood fed on the same source were not significantly different. Additionally, for both species, there was no significant difference in IVM signal detected at 0 hours or 12 hours PBF.

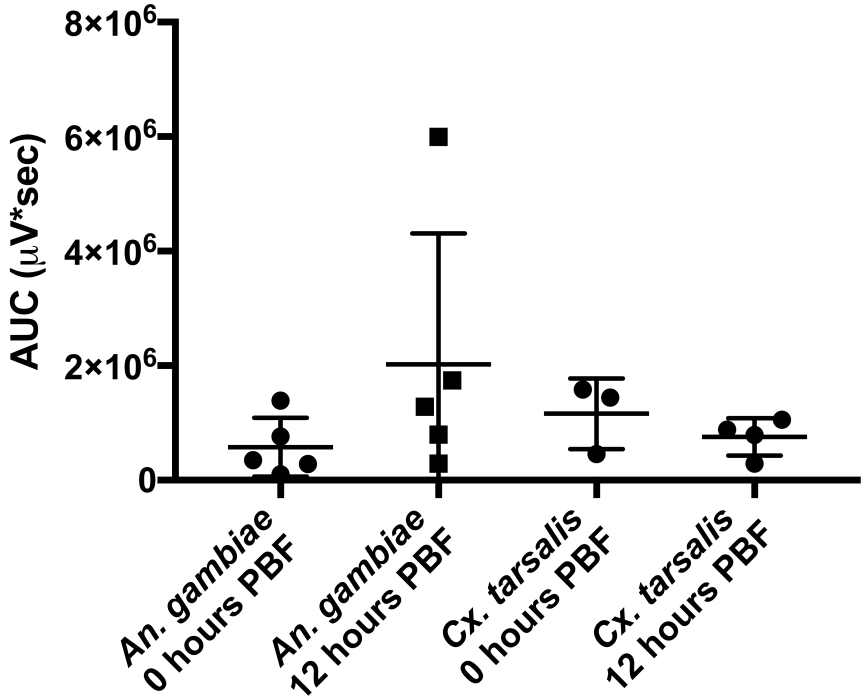

Figure 3.5 AUC from individual *An. gambiae* and *Cx. tarsalis* mosquitoes. Mosquitoes were blood fed on an artificial membrane blood meal fortified with 12.5 ng/mL IVM.

The calibration curve generated from *An. gambiae* fed serial dilutions of IVM in blood from 0.78 ng/mL to 12.5 ng/mL was linear across this range with R-square value of 0.987 (Figure 3.6). The range in AUC values reported from individual mosquitoes generally increased for higher IVM concentration blood meals. However, the COV among individual mosquitoes remained similar at 70.35% for mosquitoes ingesting the 0.78 ng/mL blood meal as compared to 66.74% for mosquitoes ingesting the 12.5 ng/mL blood meal.

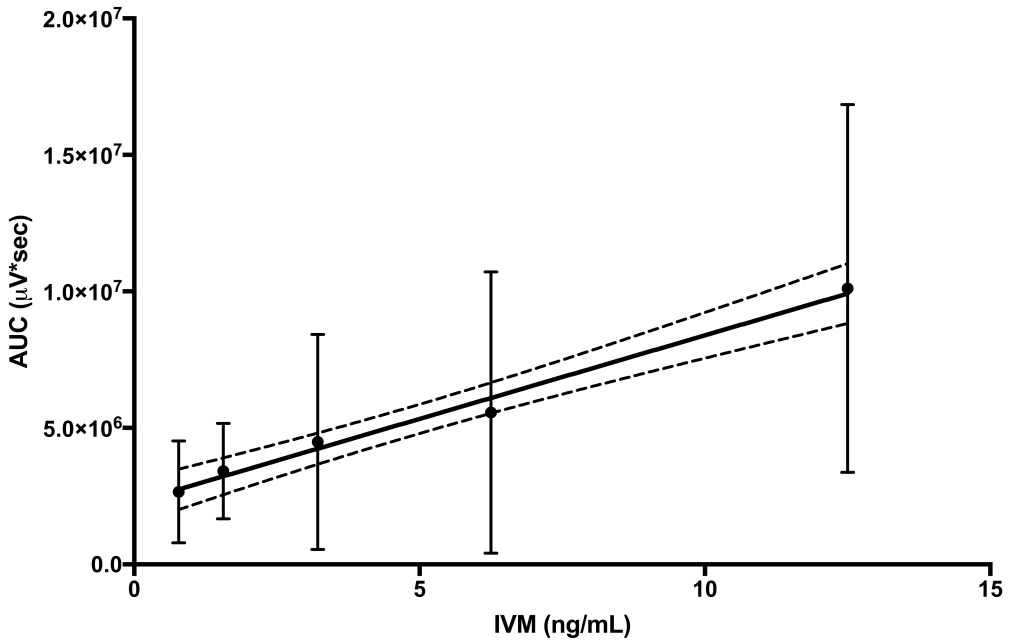

Figure 3.6 Standard curve from individual blood fed *An. gambiae* mosquitoes blood fed. Mosquitoes were blood fed on serial dilutions of IVM-fortified artificial membrane meals, ranging from 0.78 ng/mL to 12.5 ng/mL.

3.4 Discussion

This study demonstrates that IVM can be detected and quantified in individual mosquito blood meals from both *Cx. tarsalis* and *An. gambiae* mosquitoes that ingested IVM-containing blood meals. The HPLC chromatograms indicate that derivatized IVM has the same retention time for both sera and mosquito extraction and analysis, as expected (Figure 1). As expected for the fortified IVM defibrinated calf blood meal (Panel A), the chromatogram was relatively 'clean' with few other biomolecules detected, and this was reflected in the chromatogram of the mosquito fed on this blood (Panel B) as well. The chromatograms from mosquitoes fed on chicken sera directly or indirectly (Panels D & F, respectively) also reflected the extra peaks seen in the chicken serum chromatograms used in these blood feeds (Panels C & E, respectively). The early peak (blue arrowhead) is a non-specific methanol-soluble substance present in sera as it is also present in drug-free sera analysis. The later peak (green arrowhead) is likely an IVM

metabolite as it is only present in serum from an animal given IVM and is absent when pure IVM is added to a substrate. In addition, the later retention time of this peak indicates it is more nonpolar than the parent IVM drug, and more nonpolar metabolites of parent IVM have been identified to be stored in fatty tissue of cattle and sheep (10, 11). If a mosquito blood meal is an exact representation of venous serum, it would be expected that the chromatograms from the chicken's venous serum and the individual mosquito fed on the same chicken would be identical. However, this is not the case with the directly-fed mosquito chromatogram (Panel D) and its respective chicken serum chromatogram (Panel C), where the ratio of the IVM peak (black arrowhead) to the IVM metabolite peak (green arrowhead) differs between the chromatograms and it appears that the mosquito ingests a different ratio of IVM metabolite to IVM than is present in the corresponding venous serum. On the other hand, the IVM (black arrowhead) and IVM metabolite peak (green arrowhead) from the serum-replacement chromatogram (Panel E) are represented more closely in its corresponding mosquito chromatogram (Panel F). If this putative IVM metabolite has mosquitocidal activity, this could also explain the discrepancy seen between mosquito survival following a direct blood feed from a chicken as compared to a serum-replacement feed from the same chicken, as well as the difference in mosquito survival following a direct blood feed from a chicken and the predicted survival using *in vitro* LC_x as determined from IVM-spiked blood meals (Chapter 2). While further characterization is needed, this could indicate that mosquito blood meals taken directly from subdermal capillaries are not necessarily represented by venous blood analysis. This question could be explored further by comparing mosquito blood meals taken from membrane feeders containing either venous blood or capillary blood from chickens. Furthermore, *Cx. tarsalis* could be allowed to directly feed on chickens or other animals in different areas of their bodies and may vary in the amount of subcutaneous fat,

and then analyzed to determine if IVM or IVM metabolite sequestering in adipose tissue could have an effect.

There is similar variation observed among *Cx. tarsalis* blood meals taken from both artificial membrane feeders and chickens. This suggests that the variation does not result from the blood meal source, and likely occurs as a result of variable blood meal sizes ingested by individual mosquitoes and/or individual mosquito variation in blood meal metabolism. This is further supported by similar variation observed in individual *An. gambiae.* Previous studies have characterized the variable blood meal sizes ingested by mosquitoes, and how it may be influenced by factors such as mosquito age and body size (12–16). In addition, blood meal digestion is affected by body size, with larger females catabolizing blood meals more efficiently (17); however, given similar variation between 0 and 12 hours PBF, it is unlikely that this metabolism variation contributes significantly to variable IVM concentrations. This observed variation complicates the ability to precisely quantify the concentration of IVM ingested by an individual mosquito. As the standard curve for quantification does not distinguish among variable blood meal sizes or metabolism, predictions may underestimate or overestimate the IVM concentration of a blood meal. Despite this potential complication, quantifications for blood fed *Cx. tarsalis* mosquitoes fed directly on an IVM-dosed chicken had 95% confidence intervals that corresponded to the IVM serum concentration from the chicken for 86% of the mosquitoes analyzed. This ability to accurately quantify IVM chicken serum concentration based on the amount of IVM in mosquitoes directly blood fed on this chicken indicates similar IVM concentrations in subdermal capillaries on which mosquitoes feed and venous blood. This further supports the idea that the differences in survival between direct blood feeds and serum-replacement feeds are due to the mosquitocidal activity of a metabolite, rather than a

concentration gradient of IVM itself between subcutaneous adipose tissue and venous blood. However, IVM quantifications in blood fed *Cx. tarsalis* were not as accurate for mosquitoes fed on wild grackle serum in a serum-replacement feed. One explanation is that the serum and red blood cells may not have remained equally homogenous throughout the blood feed, skewing IVM consumption by mosquitoes. Another more likely explanation is that the standard curves for IVM quantification in bird sera (chapter 2) were generated using chicken serum, and therefore, may not account for any differences between chicken and grackle IVM pharmacokinetics, which may make the predicted grackle IVM serum concentration inaccurate. To address the effect of variable blood meal size on precisely quantifying IVM ingested by mosquitoes, there are biochemical and molecular methods for quantifying blood meal size, and these could conceivably be integrated into mosquito processing (18, 19). Furthermore, studies have demonstrated the use of wing length as a proxy for mosquito body size, and a ratio between wing length and blood meal size can be developed for multiple species (15). Therefore, individual wing length from mosquitoes could be analyzed in addition to their blood meals, and this could be also used to include blood meal size in IVM concentration predictions.

This method represents a crucial ability to detect IVM in individual blood meals, both immediately after blood meal ingestion and at least 12 hours following. This means that crepuscular and nocturnal feeding mosquitoes that are caught in traps overnight *(Cx. tarsalis)* and processed or aspirated the following morning *(An. gambiae)* can be assayed for the presence or absence of IVM in their blood meals. For *Culex* species and the use of endectocide-treated bird feed for arbovirus control, this is a technique to determine IVM coverage within mosquitoes in addition to birds. The IVM coverage within *Culex* populations could be an important link in

determining efficacy of the treatment, as the gold standard of reduced human disease endpoints is notably difficult to demonstrate with WNV interventions (8, 9).

For *Anopheles* spp. and the use of IVM for integrated malaria control, this detection and quantification method could be used to help answer many of the questions that remain for developing IVM as a control strategy. One such important question is determining the ideal dose and timing for IVM administrations. This question has been addressed by using pharmacokinetic and pharmacodynamics modelling paired with mosquito mortality observed from venous blood membrane feeds (ASMTH 2017 citation); however, the persistence of IVM and its concentration in mosquito blood meals following IVM administration in the field could be a useful component of these models. Furthermore, a significant parameter in assessing IVM efficacy is achieving adequate IVM coverage within the human population in order to achieve population-level effects in the vectors (1, 4, 5), and understanding IVM coverage within mosquitoes in relation to human coverage could be an integral link. Additionally, a critical component of using IVM as an antimalarial tool is the sub-lethal effects of IVM in *Anopheles spp.* that persist beyond the purely mosquitocidal effects of IVM. Sub-lethal effects of IVM that have been observed in *Anopheles* mosquitoes include inhibited flight (7), slowed digestion (6), reduced blood-feeding frequency (6), inhibited egg production (20), and inhibited *Plasmodium* sporogony (21). This method to detect and quantify sub-lethal IVM concentrations within mosquitoes could further elucidate how these sub-lethal effects contribute to IVM's effectiveness, particularly by defining the length of time following mass drug administrations that mosquitoes still ingest detectable concentrations of IVM.

In conclusion, while this study presents a preliminary proof of concept method to detect and quantify IVM in individual blood fed *Cx. tarsalis* and *An. gambiae*, it represents an

important ability determine IVM coverage within mosquito populations following IVM application to hosts, and could provide a critical link in understanding mosquito mortality dynamics in relation to IVM pharmacokinetics in humans and birds. This could provide a better understanding of using IVM as a systemic endectocide and integrated vector control strategy.

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CHAPTER 4 PILOT FIELD TRIALS TO TEST THE EFFICACY OF IVM-TREATED BIRD FEED

4.1 Introduction

Chapter 2 presented data showing that 200 mg IVM/kg of feed was a safe concentration when consumed as the sole component of a daily diet in chickens and wild-caught Eurasian-collared doves. In addition, this IVM-treated feed resulted in significantly decreased survival in *Cx. tarsalis* blood fed either directly on these IVM-fed birds or blood fed using the serum from these birds, as compared to control birds. Considering this, pilot field trials were conducted to determine if this IVM-treated bird feed would be effective in a field setting with wild birds and natural WNV transmission cycles in wild *Cx. tarsalis*.

This chapter presents two pilot field studies where IVM-treated feed is evaluated in wild birds and wild-caught mosquitoes. The 2016 field season was a preliminary trial to test IVM-treated bird feed in the field, looking for consumption and safety in wild birds and entomological endpoints. The 2017 field season used a mosquito trap to bird feeder configuration, was designed more robustly to compare three control and three IVM sites, and to compare to historical mosquito data at the same sites.

4.2 Materials and Methods

2016 Field Study

Field sites

Three field sites on CSU property were utilized. Two field sites were in northeast Fort Collins at the Agricultural Research, Development, and Education Center (ARDEC North and ARDEC South), and the third field site was located at the Environmental Learning Center (ELC) in east Fort Collins (Fig. 4.1). Each field site contained one IVM-treated bird feeder. IVM-

treated feed was used at a concentration of 200 mg IVM/kg of feed, and the feed was a mixture of white proso millet, cracked corn, and flour (47.5:47.5:5, v/v/v). IVM-treated feed was placed in feeders from the end of week 27 through week 34. IVM feed was changed daily to account for any effects of IVM degradation due to exposure, which also allowed for daily monitoring for any obvious adverse effects of IVM in local fauna.

Figure 4.1 Schematic of 2016 Field Sites. ARDEC North (A), ARDEC South (B), and ELC (C) are shown. The red pin depicts the IVM-treated bird feeder and the white circles depict mosquito traps set at 10 m (near) and 150 m (far) distances from the bird feeder.

Bird sampling

Visual bird counts were performed twice at the beginning of the trial to identify bird community composition. For three hours shortly after sunrise, each bird sighted within a 50 m

was recorded. This was done at each site on 7/6 and 7/11 for ARDEC South, 7/8 and 7/18 for ARDEC North, and 7/9 and 7/14 for ELC.

One motion-activated trail camera (Bushnell) was mounted at one feeder at each field site. The camera was triggered to take a photo whenever motion was detected. To reduce the overestimating of bird visits, consecutive time-stamped photos that did not show bird movement between frames were considered the same bird. Birds were identified to species using The Sibley Guide to Birds (1).

Mosquito sampling

Mosquitoes were collected using CO_2 -baited miniature CDC light traps (Bioquip), which were placed in linear series from the IVM bird feeder. Two mosquito traps were placed 10 m from the treated feeder and termed 'Near traps' where the IVM-treated feed would be most effective against mosquitoes, while the two mosquito traps placed 150 m from the treated feeder were considered 'Far" control traps due to their distance from the IVM-treated feeder. "Far" traps were considered control because it was expected that 150 m from the IVM-treated feeder was an adequate distance that mosquito and bird movement confounded any effects observed from IVM and therefore would be beyond the zone of control. Mosquito traps at each site were set at least once during weeks 27-31, and once during weeks 31 and 34 for ELC and ARDEC South sites in response to an outbreak of human WNV cases in east Fort Collins near the ELC site. *Cx. tarsalis* were identified and stored at -80º C for further sorting into pools for WNV screening. Up to 10 mosquitoes from each trap were dissected for parity scoring.

WNV screening was done according to established molecular protocols for WNV surveillance in Fort Collins (2). qRT-PCR was performed using the following primer sequences: forward 5' 1160-TCAGCGATCTCTCCACCAAAG 3', reverse 5' 1209-

GGGTCAGCACGTTTGTCATTG, probe 5' FAM-1186-TGCCCGACCATGGGAGAAGCTC 3' (3). Infection rates per 1000 females were calculated using the biased-corrected maximum likelihood estimate (MLE) in the Excel add-in PoolInfRate (4). When MLE could not be computed because every pool in a trap night was positive, the minimal infection rate (MIR) was calculated. The vector index (VI) was calculated by multiplying the abundance per trap night with the estimated infection rate per one female (5). Both abundance and VI were compared between IVM and control traps using Mann-Whitney U tests, as the data were not normally distributed.

2017 Field Study

Field sites

Field sites were weekly mosquito trapping sites used for Fort Collins WNV surveillance and have been maintained since 2006. Six field sites were chosen based on historical WNV surveillance data from all city trapping sites as those having the highest number of WNV-positive *Cx. tarsalis* pools since 2006, but excluding trap sites in neighborhoods that are regularly treated with adulticides or used as sentinel sites for the Colorado WNV surveillance system in the state department of health. The 6 chosen sites were all in east Fort Collins and were randomly placed into the treatment group (3 sites; mosquito traps surrounded by IVM-treated bird feed stations) or the control group (3 sites; mosquito traps surrounded by control un-treated bird feed stations). The mosquito and bird data from these sites were then compared against each other, as well as to historical data from 2006-2016 (which lacked any bird feed stations surrounding the traps). At each field site, an array of three bird feed stations was placed in an approximate triangular perimeter around the mosquito trap at a distance of 50 m. All bird feed was again changed daily throughout the trial.

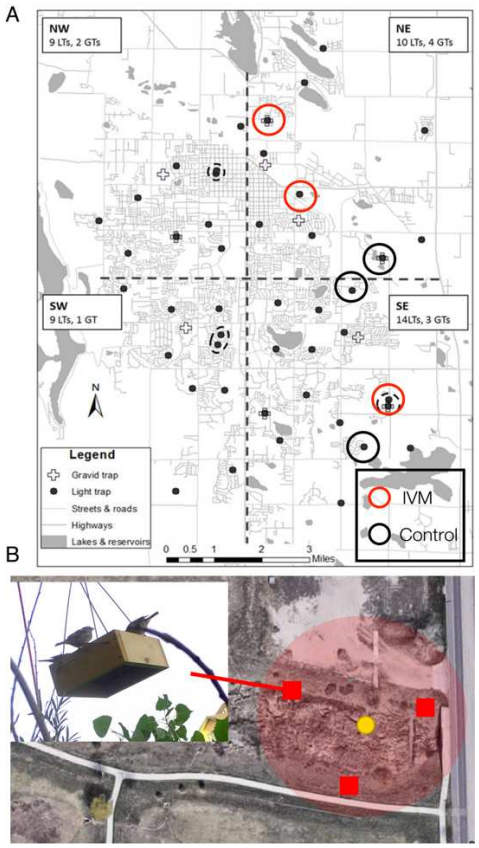

Figure 4.2 Schematic of 2017 field sites. Panel A depicts the WNV surveillance traps sites within the city of Fort Collins. The 3 chosen control (black circles) and 3 IVM sites (red circles) are shown. Black lines indicate permanent light traps. White crosses depict gravid traps. Dashed black lines indicate relocations of light traps. Each field site (B) was arranged with an array of 3 bird feeders (red squares) surrounding one mosquito trap (yellow circle).

Bird sampling

Field cameras were used to document bird visits to feeders as in 2016. However, only one of the three feeders at each site had a camera to document bird visits. Bird trapping and sampling of their blood was performed at 2 IVM sites. Birds were caught using mist nets placed approximately 10 m from an IVM-treated bird feeder. Blood was collected from netted birds using jugular venipuncture and placed into serum separator tubes. Bird sera was analyzed using HPLC-fluorescence as described in Chapter 2, and a subset of samples was analyzed using LC-MS, and control sera from house sparrows caught in Spring 2014 were used as negative controls (from the lab of Dr. Aaron Brault). Serum from one grackle was also used in a serum-

replacement blood feed with colony *Cx. tarsalis* for a survival bioassay as described in Chapter 2.

Mosquito sampling

Mosquitoes were collected weekly by Vector Disease Control International using miniature CDC light traps. Mosquitoes were sorted to species and pooled into groups of typically no more than 50. Mosquito pools were screened at CSU (Ebel lab), according to the method described for 2016.

Statistical analysis

Fisher's exact test was used to compare the number of WNV-positive and WNV-negative pools between control and treatment sites for 2017. The historical data from 2006-2016 was also analyzed using non-linear regression for comparison to 2017 data.

4.3 Results

2016 Field Study

Bird Data

All three field sites had the same species in high abundance. Swallows (not identified to species), common grackles *(Quiscalus quiscula)*, and red-winged blackbirds (*Agelaius phoeniceus*) were the most commonly visually sighted birds in abundance, respectively. Doves that are considered an important target species for this strategy were present at each site, but not observed in abundance.

Table 4.1 Bird community composition at each site. Birds sighted within a 50 m radius for three hours following sunrise were counted. Spp. indicates when birds could not be identified specifically to the species level.

Bird	ELC	ARDEC North	ARDEC South
American goldfinch *(Carduelis tristus)*		5	

House sparrow *(Passer domesticus)*			3
Blue grosbeak (*Passerina caerulea)*		1	4
Blue heron *(Ardea Herodias)*	1		
Blue jay *(Cyanocitta cristata)*	13		8
Brown headed cowbird *(Molothrus ater)*			1
Bullock's oriole *(Icterus bullockii)*	1	5	
Chickadee spp.			7
Common merganser *(Mergus merganser)*	1		
Cormorant *(Phalacrorax auritus)*	1		
Dove spp.	12	31	29
Downy woodpecker *(Picoides pubescens)*	1		
Eurasian collared dove *(Streptopelia decaocto)*	2	7	4
Flycatcher spp			1
Northern flicker *(Colaptes auratus)*	10		1
Common grackle *(Quiscalus quiscula)*	56	64	61
Ring-billed gull *(Larus delawarensis)*	1		2
American kestrel *(Falco sparverius)*		1	1
Killdeer *(Charadius vociferous)*		1	
Belted kingfisher *(Megaceryle alcyon)*	1		5
Duck spp	9	1	3
Canada goose *(Branta canadensis)*	34		
Western meadowlark *(Sturnella neglecta)*		2	10

Mourning dove *(Zenaida macroura)*	1	29	
American white pelican *(Pelecanus erythrorhynchos)*	1		
Red-winged blackbird *(Agelaius phoeniceus)*	28	96	7
Red-tailed hawk *(Buteo virginianus)*	1	1	1
American Robin *(Turdus migratorius)*	1	16	7
House sparrow *(Passer domesticus)*	1	1	1
Swallow spp.	58	24	465
Western kingbird *(Tryannus verticalis)*	1	6	6
House wren *(Troglodytes aedon)*	4		
Yellow headed blackbird *(Xanthocephalus xanthocephalus)*			3

The most common birds that visited the ARDEC South feeder as documented by the camera traps were grackles and red-winged blackbirds, although there were documented visits from both doves and small song birds that could not be identified from their photos (Table 4.2). Photos where grackles and red-winged black birds could not be distinguished were also grouped into the unidentified category. Recorded ELC camera feeder visits were mostly from by raccoons and squirrels, with one visit each by a dove and swallow (Table 4.2). The ARDEC North camera was unable to capture any bird visits due to technical difficulties.

Table 4.2 2016 Bird data as captured by field cameras. Proportions of bird and animal species visiting the feeder are given. n indicates the number of visits to the feeder counted from each species.

Animal	ARDEC South	ELC
Red-winged blackbird	16.7% (n=47)	
Grackle	64.4% (n=181)	
Tree swallow	0.4% (n=1)	

Unidentified	18.5% (n=52)	4.6% (n=2)
Squirrel		93.1% (n=40)
Raccoon		2.3% (n=1)

Mosquito data

Figure 4.3 depicts the abundance for *Cx. tarsalis* for all 2016 field sites. There was no significant difference in abundance for "near" and "far" mosquito traps at any of the three field sites. In addition, while mosquitoes of other species were not counted, there was no obvious differences in *Cx. tarsalis* in relative to other mosquitoes at "near" and "far" traps at any of the sites observed when processing mosquito collections.

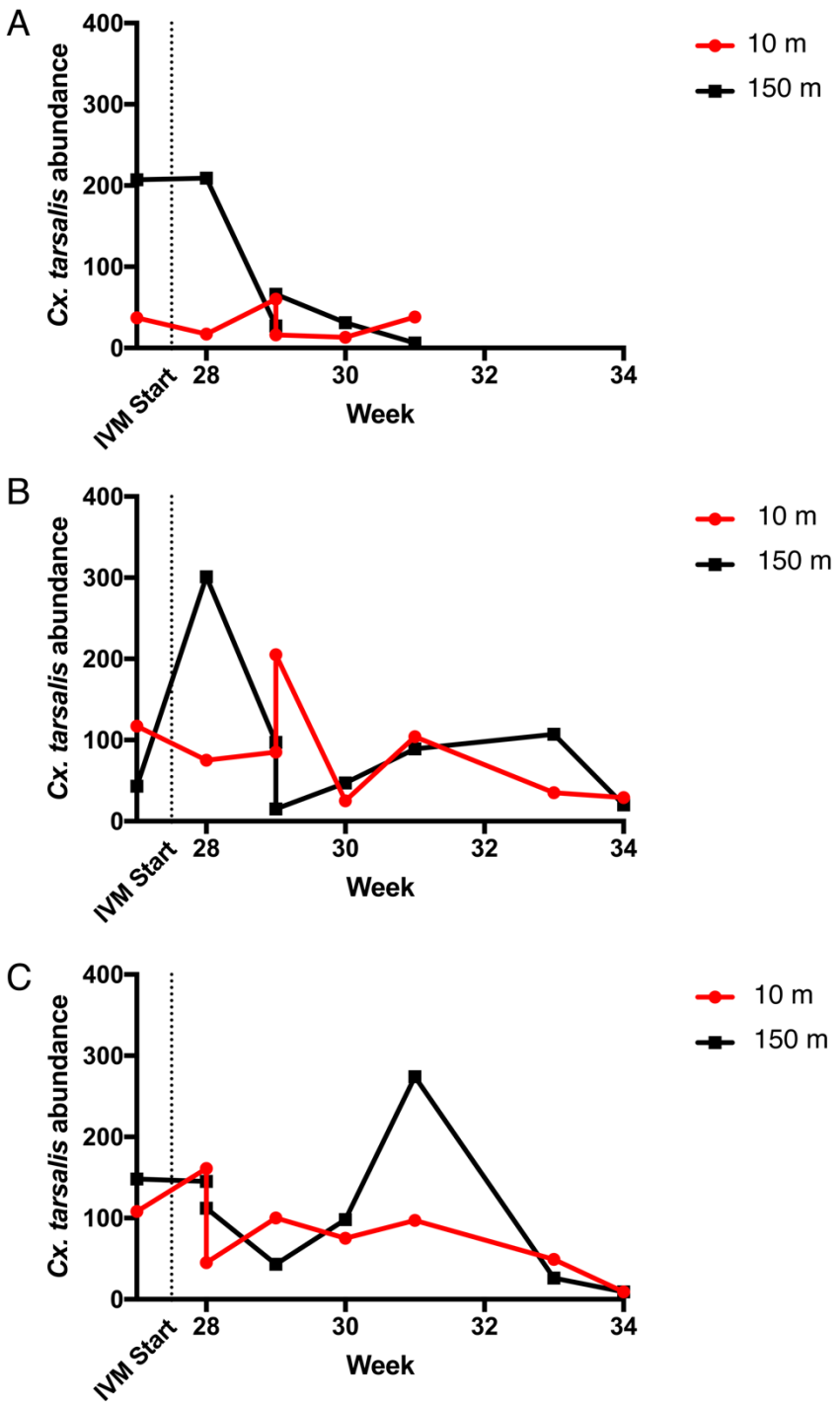

Figure 4.3 *Cx. tarsalis* abundance at ARDEC North (A), ARDEC South (B), and ELC (C) sites over the 2016 season. Dotted line indicates when IVM-treated feed was started.

There was no significant difference in parity rates between IVM and control mosquito traps (Figure 4.4).

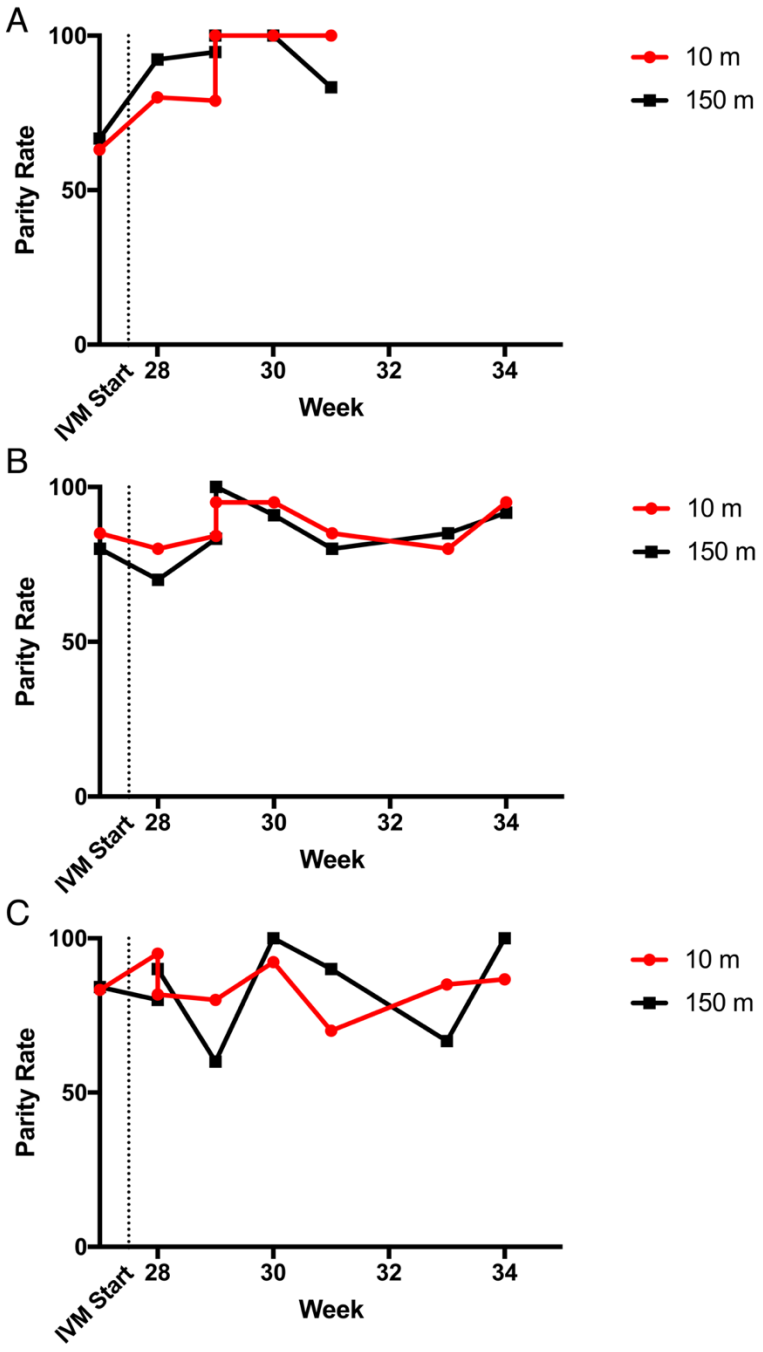

Figure 4.4 Proportion of parous *Cx. tarsalis* from mosquito traps at ARDEC North (A), ARDEC South (B), and ELC (C) over the 2016 field study. Dotted line indicates when IVM-treated feed was started for the trial.

ARDEC North had no WNV-positive pools at either control or IVM traps, thus the VI remained zero for the entire season. ARDEC South showed a marginally significant difference in number of WNV-positive pools for control compared to IVM traps (p=0.0915), but there was a trend of "far" traps having WNV-positive pools for 4 weeks and increasing over the trial, while there was only one week with WNV-positive pools for the "near" traps at the end of the trial (wk. 34). The ELC site also showed no significant difference (p=0.6653) in number of WNV-positive pools between "near" and "far" mosquito traps, but again showed a trend of "far" traps having a more sustained positive VI over the trial as compared to "near" IVM traps, which had positive pools in only weeks 29 and 34.

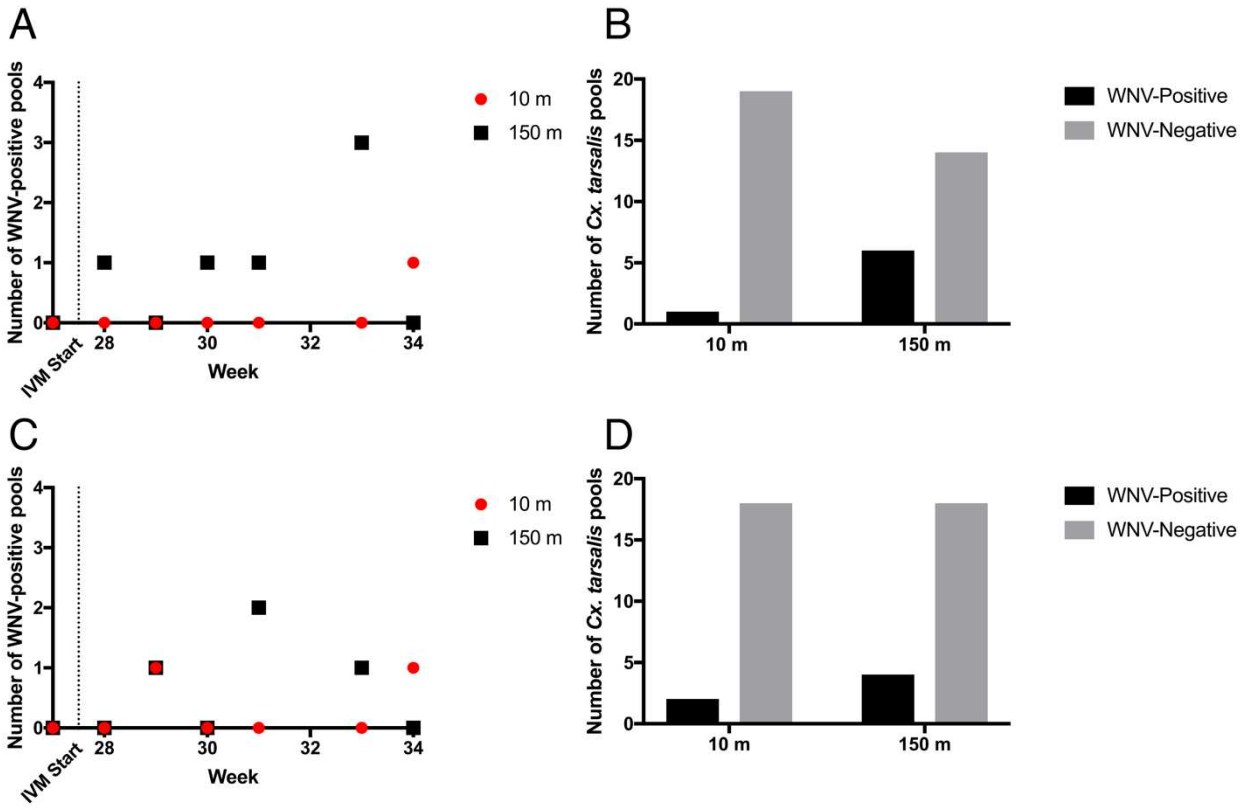

Figure 4.5 WNV-positive pools over time for ARDEC South (A) and ELC (C) sites during the 2016 field season. Dotted line indicates when IVM-treated feed was placed into bird feeders. Proportion of WNV-positive and WNV-negative pools is shown for ARDEC South (B) and ELC (D).

2017 Field Study

Bird data

Table 4.3 Bird visits as documented by field cameras on bird feeders. Percentages indicate proportion of visits composed of indicated species and n indicates number of documented visits made by the indicated species.

Animal	Control Sites	IVM Sites
Grackle	92.5% (n=1219)	22.8% (n=31)
Blue Jay	0.15% (n=2)	22.8% (n=31)
Sagebrush sparrow	0.075% (n=2)	1.5% (n=2)
Squirrel	1.3% (n=15)	19.9% (n=27)
Raccoons	0.5% (n=7)	
House sparrow	0.075% (n=1)	0.74% (n=1)
Black-capped chickadee	2.5% (n=33)	6.6% (n=9)
Unidentified	2.8% (n=37)	3.7% (n=5)
Bushtit		2.9% (n=4)
Brewer's sparrow		19.1% (n=26)

Bird visits to IVM feeders at all sites were heavily dominated by grackles with infrequent visits by house (*Passer domesticus)* and sagebrush sparrows (*Artemisiospiza nevadensis)* and black-capped chickadees (*Poecile atricapillus)*. There were also two visits by blue jays *(Cyanocitta cristata)*, and a few other birds could not be identified from their positions in the photographs. A more homogenous mix of grackles, house and brewers *(Spizella breweri)* sparrows, blue jays, black-capped chickadees, bushtits, and squirrels visited control feeders.

Ten grackles and 5 sparrows were caught over 4 mornings of sampling on 8/30, 9/2, 9/3, and 9/7. The sera were analyzed for IVM using HPLC-fluorescence. Most birds had been observed feeding from the IVM-treated feeder immediately preceding mist net capture. 9 grackles and 4 sparrows (87% of tested sera) had detectable levels of IVM within their serum, and the negative control sparrow serum from 2014 had no detectable IVM. LC-MS also confirmed the presence of IVM within one grackle serum sample (Figure 4.6).

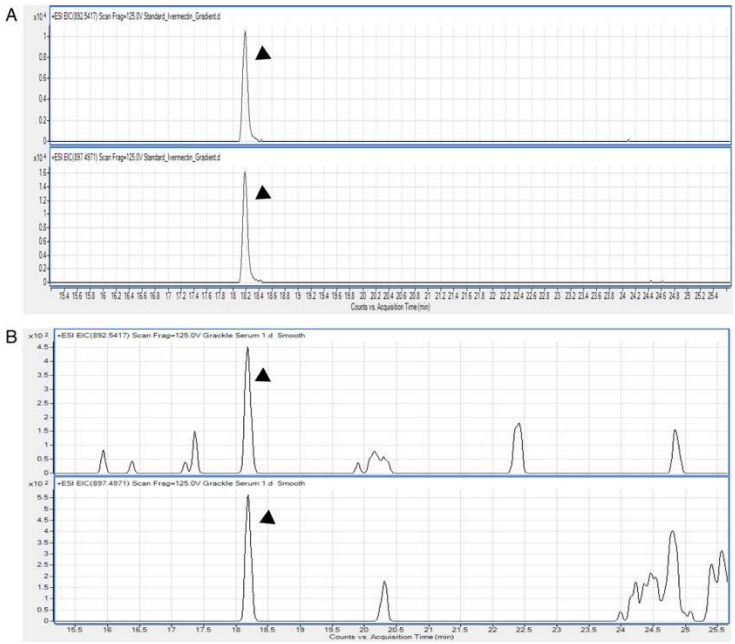

Figure 4.6 LC-MS confirmation of IVM in wild-caught grackle serum. Panel A depicts IVM standard in acetonitrile and Panel B depicts wild-caught grackle serum. IVM peak is delineated with a black arrowhead.

To confirm bioactivity, the serum from this grackle was used in a serum-replacement blood feed for a *Cx. tarsalis* bioassay, and there was significant difference ($p<0.0001$) in survival as compared to mosquitoes fed on control defibrinated calf blood (Figure 4.7).

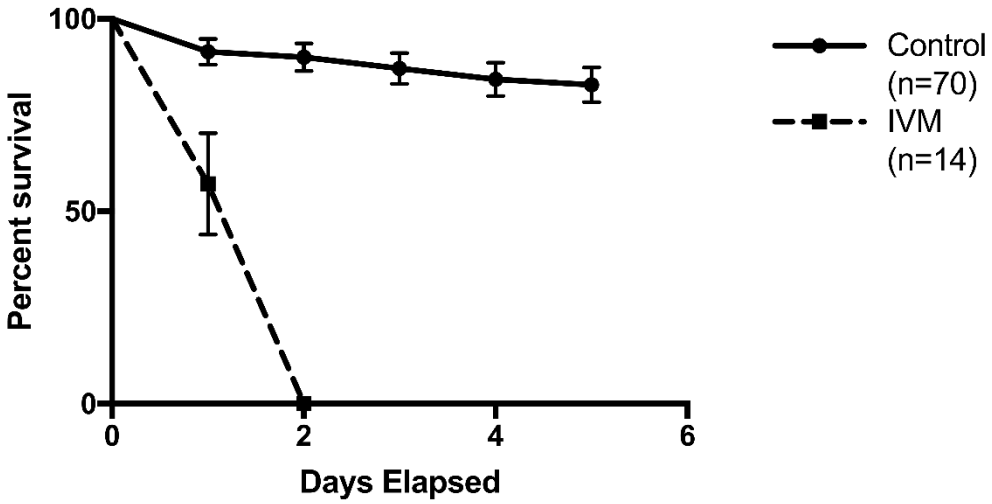

Figure 4.7 *Cx. tarsalis* survival following a serum-replacement blood feed using serum from a wild-caught grackle in which IVM was detected (dotted line) compared to control calf blood (solid line).

Mosquito Data

It is not possible to definitively conclude that a difference exists between control and IVM sites using data from a single season. However, there was a trend in the number of WNV-positive pools between control and IVM sites, where control sites had more WNV-positive pools that occurred earlier as compared to treatment sites, suggesting a possible effect from IVM (Figure 4.8).

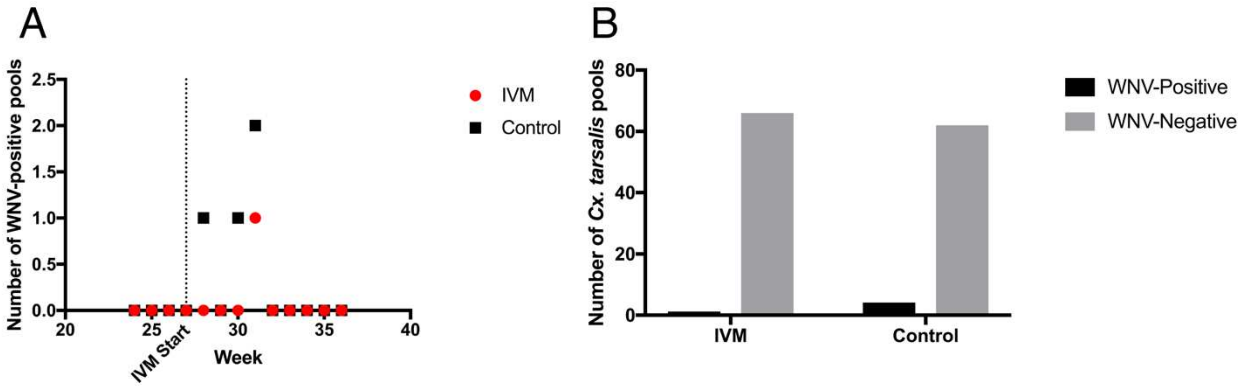

Figure 4.8 Number of WNV-positive *Cx. tarsalis* pools over the 2017 season is shown in panel A. Dotted line indicates the start of IVM feed. Panel B depicts the proportion of WNV-positive and WNV-negative pools at control and treatment sites.

Figure 4.9 shows the abundance for 2017 control and IVM sites in comparison to a regression of the historical data from these traps. There was no observable difference in 2017 *Cx. tarsalis* abundance between control and treatment (Figure 4.9).

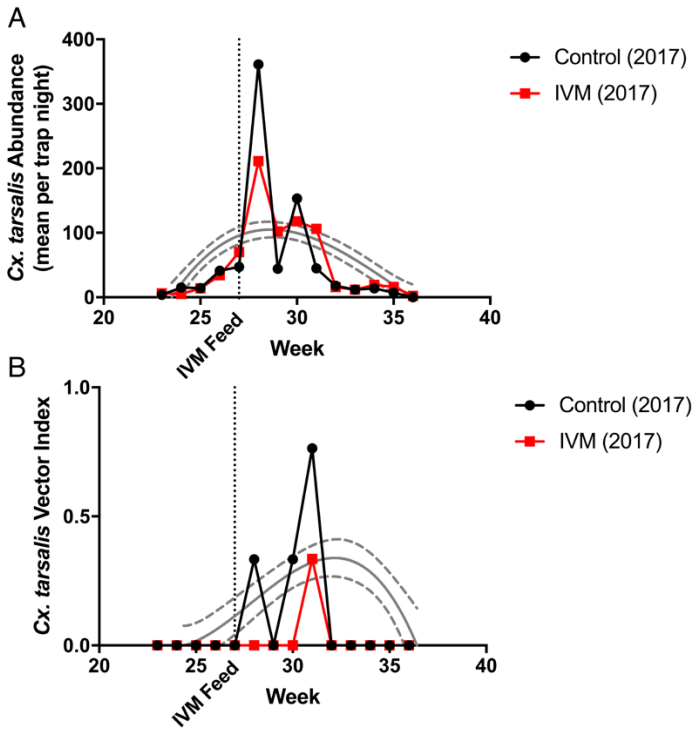

Figure 4.9 *Cx. tarsalis* abundance (A) and vector index (B) for 2017 field sites. Historical data from the same sites (2006-2017) were fit to non-linear regression lines (solid gray) with 95% Cis (dashed gray lines), and 2017 data were plotted as points. Dotted lines indicate the start of IVM-treated feed at IVM sites.

4.4 Discussion

This chapter presents promising results from two separate trials of IVM-treated bird feed during the summers of 2016 and 2017. Results from 2016 show that wild birds frequently visit the IVM-treated feeders. In addition, there was an observable trend where "far" traps that are expected to be beyond the zone of control had higher VI compared to "near" traps at both ELC and ARDEC South sites. Results from the 2017 study continued to be promising, where birds were again visiting IVM-treated feeders and IVM could be detected in the sera of birds sampled by IVM feeders. There was also a trend of higher VI for the control sites compared to IVM sites for the 2017 season.

The 2016 study was designed as a pilot trial for IVM-treated feed in a natural environment, thusly, the study was not designed with control and IVM-treated feeders. There

were three IVM-treated feeders, and mosquito traps were set at 10 m and 150 m distances. It was expected that 150 m would be a large enough distance that bird and mosquito movement would confound any effects of IVM treated feed, while an effect from IVM might be observable at 10 m from the feeder.

Visual bird counts at all three 2016 field sites showed diverse communities, and the dove and passerines we expected to target were present. Field camera showed that ARDEC South sites had bird visits mostly from grackles and red-winged blackbirds, while ELC sites were mostly from mammals, indicating that our targeted birds were not making frequent visits to the IVM-treated feeders.

An important component of the entire strategy of using IVM as a systemic endectocide to control WNV transmission is the potential for shifting the mosquito population age structure in a zone around IVM-treatment feed stations from older, infectious mosquitoes to younger, non-infectious mosquitoes. This has been modeled, as well as observed with empirical data, in trials testing IVM for malaria transmission control (6, 7). The conventional use of parity rates to score a mosquito as parous (having laid eggs) or nulliparous (not having laid eggs) relies on noticeable changes in tracheole skein coiling on the ovaries following egg clutch development. Using parity as a parameter for age is dependent on the fact that a mosquito takes a blood meal before developing a batch of eggs (gonotrophic concordance), and that this gonotrophic cycle lasts several days. Therefore, a younger, nulliparous mosquito has not yet taken a blood meal and had an opportunity to become infectious. However, there was no significant difference between parity rates in 2016 for *Cx. tarsalis* caught at traps close to the treated bird feeders as compared to farther away. Given that rates of parous *Cx. tarsalis* were consistently high across all traps, it is likely that autogeny, or the ability to develop a batch of eggs without imbibing a blood meal,

resulted in high baseline rates of parity without regard to age. This observation is supported by studies that have found high rates of autogeny in field *Cx. tarsalis* in California (8, 9). Due to this complication of autogeny in *Cx. tarsalis,* an alternative measure of age should be used to determine the age structure of the mosquito population.

For the 2016 study, there was no significant difference in mosquito abundance between "near" and "far" traps at any of the three field sites. However, there was an observable trend for VI where "near" traps had lower VI as compared to "far" traps throughout the season. It is likely that our study design was limited by confounding factors such as bird and mosquito movement. It is unlikely that an effect from IVM would be observed if birds were ingesting IVM-treated feed at the feeder but then getting bitten by *Cx. tarsalis* in an area not sampled by our mosquito trap design. In addition, our study was not designed with high statistical power.

The 2017 field study was designed more robustly with both control sites with control feed and IVM sites with IVM-treated feed. The use of three bird feeders around one mosquito trap also made it more likely to control for bird movement in relation to the mosquito trap in comparison to 2016. In addition, there was historical WNV data from these specific sites for comparison.

Trail camera data from 2017 indicated that grackles were predominantly visiting our IVM-treated feeders, while control feeders were visited mostly by grackles, blue jays, brewer's sparrows, and squirrels. However, as there was only one trail camera at each field site for 2017, it is likely that the single trail camera did not fully document visits to other feeders at the field site. This could be especially true for feeders where dominance by grackles and other aggressive bird species may have driven other species to frequent other feeders at the same site without a trail camera to document visits. Camera placement was limited to areas where there were trees

where a feeder could be hung, and a camera locked to a tree across from the feeder. This may have biased the camera data away from bird species that feed in open space as compared to among the trees. This limitation of the field camera data is illustrated by the detection of IVM in sparrows caught by mist net, but no documentation of sparrow visits on the trail camera for this specific IVM site. It is especially promising that a majority of the birds tested had detectable levels of IVM within their sera, indicating that there was an unexpectedly high coverage of IVM in captured birds. The placement of mist nets at roughly a 10 m distance from an IVM feeder likely biased the birds sampled towards birds that visited the feeder, so future studies should sample birds at a wider radius. This is an important component of determining the efficacy of this strategy and should be coupled with detection of IVM in wild-caught blood fed *Cx. tarsalis* in future field seasons.

Mosquito data for the 2017 study seemed to indicate a possible effect from the IVM-treated bird feed, although it is difficult to draw definitive conclusions from one field season. The VI among mosquitoes captured at IVM-treatment sites was lower than VI among mosquitoes captured at untreated control sites over the 2017 field season. However, neither VI nor abundance from IVM sites were statistically different from untreated historical data. It is important to note that comparing one year of IVM treatment data at these sites to 10 years of historical data would bias the sampling and could overwhelm any effects from IVM. Future directions should include more control and IVM sites over multiple WNV seasons.

Future field trials of IVM-treated bird feed should be adjusted following the observations that target bird species are not frequently visiting IVM-treated bird feeders. An important direction would be to determine the blood meal preferences for *Cx. tarsalis* within the study area. The decision to preferentially target Eurasian collared doves and passerine birds was based on

Kent et. al demonstrating that a consistently large proportion of *Cx. tarsalis* blood meals are derived from these sources in nearby Weld County (10, 11). However, this study may not translate accordingly to the more urban Fort Collins area, especially given the extensive housing and land development in the area over the past 10 years. Following this, bird feed composition could also be optimized to target preferred bird species around each mosquito trapping site. Our current bird feed (an equal mixture of cracked corn and millet) was used because our characterization of IVM-treated feed was in cracked corn and millet has been shown to be readily consumed by doves (12). However, choice studies to determine the ideal mixture could be performed with wild-caught birds of the appropriate species. In addition, the bird feeder design itself could be optimized to target preferred species. Platform tray feeders had been chosen to allow for adequate access for a wide variety of birds and feeding styles, but feeder design could be styled towards target bird species, while also excluding undesired visits from other animal and bird species.

This chapter presented data from two field trials that explored the efficacy of IVM-treated feed in a field setting with natural WNV transmission cycles in wild birds and field *Cx. tarsalis.* It demonstrated that important mosquito and bird sampling necessary to evaluate the effectiveness of IVM-treated feed could be performed with promising results when the data were analyzed. This indicates that more extensive field trials with higher statistical power should be conducted to further explore the use of IVM-treated feed for control of WNV transmission.

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CHAPTER 5 DISCUSSION

5.1 Conclusions

This study presents the development of IVM-treated bird feed for the control of WNV transmission as well as a pilot field trials to test the use of treated feed as a control strategy. The highest WNV disease incidence occurs along the Great Plains region of the United States (1), as the irrigated agriculture provides a supportive habitat for the main WNV enzootic and bridge vector of the region, *Culex tarsalis* (2). WNV is maintained in an enzootic cycle between *Culex* mosquitoes and avian hosts, therefore, blood meals by *Cx. tarsalis* from preferred avian species may be utilized to selectively target adult females through their blood feeding behavior. Given that the majority of *Cx. tarsalis* blood meals on the northern Colorado plains may come from doves and passerine species during the WNV transmission season (3), effective targeting of these preferred hosts with endectocide-treated bird feed could result in control of WNV transmission.

Aim 1 was addressed in Chapter 1 where the *in vivo* effects of multiple IVM formulations were characterized in *Cx. tarsalis* and birds. Chapter 1 illustrated that following 3 days of IVM feed consumption at 200 mg IVM/kg of feed, chickens achieved concentrations of IVM within their blood that were lethal to *Cx. tarsalis* in either a direct blood feed or a serum-replacement blood feed. *Cx. tarsalis* directly blood fed on wild-caught Eurasian collared doves given IVM feed also had decreased survivorship compared to control doves. These lethal IVM concentrations dissipated quickly following IVM feed withdrawal, and by 2 days post-withdrawal, *Cx. tarsalis* no longer had significantly decreased survival following blood feeding on IVM-fed chickens as compared to control chickens and this was corroborated by IVM sera concentrations that were generally below detectable levels. Neither IVM feed formulation nor

the time for which it was administered affected mosquitocidal efficacy, IVM serum concentrations, or IVM serum $t_{1/2}$. In addition, the higher correlation between IVM serum concentration and cumulative mosquito mortality for serum-replacement feeds than observed for direct blood feeds suggests that venous serum analysis may not be an exact representation of direct blood feeds.

Chapter 2 also introduced the experimental method of using serum-replacement feeds to assess the systemic, mosquitocidal effects of IVM when given to live animals. Serum-replacement feeds using venous blood drawn from birds may not be an exact biological representation of direct feeds on live animals. This is based on observations of decreased survivorship from mosquitoes directly blood fed on chickens as compared to serum-replacement feeds using venous serum from the same chickens. As one explanation for this observed discrepancy is the presence of a mosquitocidal IVM metabolite sequestered in fatty tissue, serum-replacement feeds using capillary blood rather than venous blood should be compared to direct blood feeds from the same chicken. However, serum-replacement feeds still remain an important tool for testing IVM efficacy against mosquitoes from live animals, even if they are not an exact biological representation of direct blood feeds. Direct blood feeds on live animals using *Cx. tarsalis* usually result in extremely low numbers of successfully blood fed mosquitoes, and therefore require close coordination of abundant mosquito colony numbers with the timing of IVM administration in animals. Serum-replacement blood feeds address these issues as they result in high rates of successful blood feeding and allow for freezing of serum rather than coordinating the timing of IVM in live animals with mosquitoes. Furthermore, serum-replacement feeds allow for the testing of large numbers of wild bird serum samples for mosquitocidal efficacy, which would not be feasible if every test were a live feed of mosquitoes

on a wild bird. While mosquito blood feeds done directly on live IVM-treated animals remain important, serum-replacement blood feeds are an important supplemental tool.

Aim 2 was addressed in Chapters 2 and 3 where IVM pharmacokinetics in chickens and mosquitoes were studied. In Chapter 2, HPLC-fluorescence was used to quantify IVM chicken serum concentrations following IVM consumption and for two days following withdrawal and analyzed in relation to mosquito bioassay data. Chapter 3 focused primarily on characterization of IVM from blood fed mosquitoes. IVM could be detected and quantified from both blood fed *Cx. tarsalis* and *An. gambiae* at 0 and 12 hours PBF, allowing for detection in field settings. This ability to detect IVM at 12 hours PBF is particularly important as it allows for determination of IVM coverage within a target wild mosquito population that has imbibed a blood meal the evening before and then is sampled the morning following for analysis. IVM quantification from *Cx. tarsalis* directly blood fed on an IVM-dosed chicken was fairly accurate. IVM quantification for *Cx. tarsalis* blood fed on a serum-replacement using serum from a wild grackle caught near a IVM-treated feed was less accurate; this is likely due to differences between grackle and chicken pharmacokinetics as chicken serum was used to generate the bird IVM serum standard curve. There were no statistically significant differences in IVM concentrations between 0 and 12 hours PBF for either *Cx. tarsalis* and *An. gambiae* nor between the mosquito species themselves. There was a wide range of variation in IVM signal detected from individual blood fed mosquitoes blood fed on the same blood meal source, and this is likely due to variations in mosquito blood meal size. Comparison of blood fed mosquitoes from direct blood feeds and serum-replacement feeds to their respective venous serum chromatograms suggested that an IVM metabolite with mosquitocidal activity could be responsible for the discrepancy in mosquito bioassay data between these two methods.

Aim 3 was presented in Chapter 4 where the results from two pilot field trials were presented. The field study in 2016 was designed with three field sites that each had one IVM bird feeder with mosquito traps set at 10 m and 150 m distances from the feeder, which may not have been adequate distances to account for bird and mosquito movement. ARDEC bird feeder visits were by mostly grackles and red-winged blackbirds with a few visits from doves and unidentifiable song birds, while ELC visits were overwhelmingly from squirrels and raccoons. There was no difference in parity between control and IVM mosquito traps at any of the sites, and autogeny in the *Cx. tarsalis* population was likely a confounding factor that contributed to consistently high parity rates. There was also no difference in abundance between control and IVM *Cx. tarsalis* traps at any of the three 2016 field sites. ARDEC North had no WNV-positive pools, which did not allow for a comparison of IVM and control mosquito trap VI rates. There was no significant difference in VI between control and IVM mosquito traps for either ARDEC South and ELC sites, but there was a general trend of control mosquito traps having higher VI compared to IVM mosquito traps over the season. For the 2017 field season, visits to feeders at IVM sites were predominantly grackles, followed by infrequent visits of house sparrows, sagebrush sparrows, and black-capped chickadees. Bird visits to feeders at control sites were a more homogenous mixture of grackles, house sparrows, Brewer's sparrows, blue jays, black-capped chickadees, and squirrels. IVM was detected in 87% of birds caught using mist nets at IVM sites. IVM was further confirmed in one grackle sample using LC-MS, and a serum-replacement blood feed using this serum also resulted in significantly decreased survival in mosquitoes compared to mosquitoes fed on control calf blood. Like 2016, there was a trend of lower VI between IVM sites compared to control sites in 2017; however, it is difficult to definitively conclude from single season of data.

5.2 Future Directions

Building upon the research presented in this dissertation is crucial for developing IVM as a systemic endectocide for control of WNV transmission and will also provide further insight to its use as supplemental control strategy for malaria transmission.

Characterization of the potentially mosquitocidal metabolite of IVM could address observations of increased IVM efficacy than expected from LC_x as determined by *in vitro* membrane feeds as well as a "post-ivermectin effect", where increased mosquito mortality is still observed following feeding on treated volunteers past the point when IVM is detectable in plasma. This could also address the observed differences in IVM efficacy where mosquito mortality from a direct blood feed from an IVM-treated animal is higher than mosquito mortality following a serum-replacement blood feed from the same animal. As an extraction method for this metabolite from serum has already been optimized and it has been characterized to be more nonpolar than IVM, HPLC can be used to isolate this metabolite from serum of IVM-treated animals and *in vitro* membrane blood feeds can be used to determine if it has mosquitocidal activity. Additionally, the structure of the metabolite could be further characterized using mass spectrometry and the metabolite could be commercially synthesized for future analysis. Future studies could determine the $t_{1/2}$ of this metabolite with IVM-treated animals, its deposition in tissues of animals treated with IVM, and any synergy with parent IVM.

Another future direction of this work includes the optimization of an environmentally stable formulation of IVM in bird feed. This would extend the time for the IVM-treated bird feed could remain in the feeder before needing to be replaced due to heat and UV degradation of IVM, and making it a more convenient for deployment as a WNV control strategy. Another non-mutually exclusive direction would be making the IVM more long-lasting within a bird that

consumes IVM-treated feed so less frequent visits to a treated bird feeder are required, extending the efficacy of IVM. Slow-release formulations of IVM given to humans are currently being explored for malaria control (4–6). While there would understandably be safety and environmental concerns to be addressed with a slow-release IVM formulation, they are currently available to treat sheep and slow-release eprinomectin, a closely-related drug to ivermectin, is available for cattle.

One limitation of this study is that it lacks an extensive exploration of IVM toxicity in birds. While chickens have been reported to have had substantially higher IVM plasma concentrations than observed in our study without any adverse effects (7), that report did not examine the possibility of long-term effects of IVM in birds, and neither did our studies presented here. One study has observed longer-term administration of avermectin, of which ivermectin is a safer derivative, resulted in signs of neurological depression in pigeons given 20 mg avermectin/kg of feed for 60 days and more obvious signs of neurotoxicity in pigeons given 60 mg avermectin/kg of feed for 30 days (8). While it is unlikely that a bird would visit our IVM-treated feeder daily as well as consume IVM-treated feed as its sole diet for the WNV season, a more nuanced exploration of IVM neurotoxicity in birds should be done. This could include looking for some of the signs of neurotoxicity observed in pigeons following avermectin exposure including oxidative stress responses and histopathological changes in brain tissue (9).

Critically important to this strategy is adequate IVM coverage within the mosquito population in order to see effects in entomological or human and animal disease endpoints. This would require the application of the HPLC-fluorescence assay discussed in Chapter 3 to detect the presence of IVM in wild-caught, blood fed mosquitoes while IVM-treated feed is available for consumption by wild birds. In addition, this detection could be paired with quantification of

IVM in blood fed mosquitoes to determine the contribution of lethal and sublethal IVM effects within the mosquito population. When this is paired with assaying for the prevalence of IVM coverage within the bird community, IVM coverage in mosquitoes could be correlated to the IVM coverage within birds.

The results presented in this study are promising for the development of IVM-treated bird feed for the control of WNV transmission. The efficacy of IVM-treated feed was evaluated in two trials using a field setting with natural WNV transmission cycles in wild birds and *Cx. tarsalis,* but should be followed up with field seasons with many control and IVM sites to allow for a robust analysis of IVM effects. This study introduces the novel concept of using systemic endectocides for controlling WNV transmission, and this concept could be explored for other arboviruses.

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