INSECTS ASSOCIATED WITH SHALLOW-TRENCH COMPOSTING OF SWINE CARCASSES FROM A SIMULATED AFRICAN SWINE FEVER DISEASE OUTBREAK AND POTENTIAL FOR VIRUS TRANSMISSION

By

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Abstract: African Swine Fever (ASF) Virus is a double-stranded DNA virus that causes a highly contagious hemorrhagic disease with almost 100% mortality in swine. Soft ticks (Argasidae), swine lice (*Haematopinus suis*), and stable flies (*Stomoxys calcitrans*) are competent insect vectors of the virus. These studies focused on insects associated with the burial site of pig carcasses and the potential of identified insects to transmit ASF from burial sites. For the first study, shallow-trench composting of pig carcasses was examined, with 50 pigs each buried under soil in two trenches, 50m long × 0.6m deep × 2m wide, lined with 0.3m of wood chips. Both femurs were inoculated, at time of burial, with swine pox virus to model ASFV infection. Pitfall and malaise traps were used to collect attracted insects. Traps were sampled every 2-3 days August-October, once-a-month November-March, and every other week from April-September. All insects were identified to family with further classification of Coleoptera and Diptera specimens to genus or species. A total of 22,730 arthropods were identified. The most common fly family was Calliphoridae and the beetle family Silphidae was found only at the trench site. To assess persistence of swine pox virus and its movement by insects, insects within the animal mortality trenches were collected post-burial after 1, 2, and 3 weeks, and at 1,2,4,6 and 12 months. The second study examined house flies vector competence for three surrogate viruses: Swinepox (SwPV), Bovine Viral Diarrhea Virus (BVDV), and Senecavirus A (SVA). Twenty-four flies were exposed to one virus for 1 hour and at 0, 6, 12, 24, and 48 hours post exposure the flies were collected. Then flies were tested for presence of DNA/RNA and viable virus. All flies exposed to SVA, males at hour 0 for BVDV, and SwPV females at hour 6 had viable virus. Proven vectors of ASFV were found at the animal mortality trenches with an expected emergence date two days after the last time viable virus was collected from the trenches. House flies were shown to be able to hold viable SwPV, surrogate for ASFV, for 6 hours post exposure.

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CHAPTER I

Literature Review

Animal Mortality Management

Mass mortality events happen in animal facilities without notice. Some of the causes are natural disasters like tornadoes, earthquakes, or hurricanes and others are caused by disease outbreaks. In the event of mass animal mortality, management of the carcasses can be handled in different ways. The most common disposal methods are landfills, incineration, burial, and composting (Costa and Akdeniz 2019). Landfills are usually less costly but give access to scavenging animals. Incineration is considered to destroy all infective agents but the transportation of infected animals to off farm locations raises concerns (Gwyther et al. 2011). For example, the transportation of animals in the United Kingdom was found to facilitate the spread of foot and mouth disease. Another concern is a lag time between the death of an animal and the collection which can be a hazard if the carcass is not stored properly (Gwyther et al. 2011). Burial of the animals has been a common farm practice of handling mass mortality. However, concerns about the potential infectious agents leaching out and entering the human or animal food chain has led to the banning of the practice in the European Union (Gwyther et al., 2011).

Composting is the preferred method of carcass management according to biosecurity agents in the United States of America, Canada, Australia, and New Zealand (Wilkerson 2007).

Composting inactivates the pathogens by heat and time. Most pathogens are inactivated by exposure to an average temperature of 50-60^{O}C for several days (Epstein 2017; Costa and Akdeniz 2019). However, some pathogens, including *Clostridium, Bacillus antracis,* and Bovine spongiform encephalopathy (BSE), are not suitable for composting because no inactivation occurs. Other pathogen like Aphto virus, which is the causal agent of foot and mouth disease, have conflicting data on whether composting inactivates them (Farnke-whittle and Insam 2013). Area and size of the carcasses can be an issue for composting and carcasses (>50kg) have to be in a single layer (Wilkerson 2007). A new method being tested is shallow trench composting. It combines both burial and composting to enhance the processes from both these types of mortality managements techniques. Each method of carcass disposal has advantages and disadvantages but one common issue with all methods is that insects gain access to the carcasses.

Insect Associated with Decomposing Animals

Each stage of decomposition has its own entomological fauna. The most common insects to visit the fresh carcasses are filth flies. The three main filth fly families are Calliphoridae, Sarcophagidae, and Muscidae. Beetles such as Staphylinidae, Silphidae, and Dermestidae will also visit the carcasses at later times (Rosa et al. 2011). Calliphorid flies are usually the first and most dominant taxa on carrion (Dubie and Talley 2017). Some studies have found that Muscidae are the first to reach buried carcasses (Gunn and bird 2011, Amendt et al. 2010). Insects may feed on the carcasses directly or prey on flies that have colonized the carcass first (Rosa et al. 2011, Dubie and Talley 2017). The deeper the carcass is buried the longer it takes for insects to colonize it. Pastula and Merritt (2013) found it took 2 days longer for flies to colonize a carcasses buried at 60cm compared to 30cm. flies activity decreases in cold months. Turner and Wiltshire (1999) found that buried pig carcasses place in December were not colonized by flies until scavengers disturbed the site in April. Calliphoridae flies have been show to reach buried remains at 10cm, but how also be shown to lay egg on the surface to allow larvae to crawl down to the carcass

(Gunn and Bird 2011, Amendt et al. 2010). Dubie and Talley (2017) found that 49% of all samples collected from a bovine composting area were Calliphoridae. Similarly, a study on Louisiana wildlife found the main species was the hairy maggot blow fly (*Chryomya rufifacies*) which is a Calliphorid species (Watson and Carlton 2005). These insects use the available food source to complete their life cycle and they also help decompose the carcasses (Mashaly et al. 2019).

While filth flies are most commonly associated with bacterial pathogens there is some evidence that they could play a role in mechanical transmission of viral pathogens (Nayduch and Burrus 2017; Nayduch et al. 2002; Khamesipour et al. 2018). In lab experiments house flies have been shown to carry pathogens including, Senecavirus A, *Cryptosporidium parvum*, and *Bacillus anthrax* (Khamesipour et al. 2018; Graczyk et al. 1999; Fasanella et al. 2010). Nayduch and Burrus (2016) found that some bacteria can survive the digestive tract of house flies. They also found that bacteria can exchange genetic material while in the fly. This allows the fly to disseminated bacteria through excreta to new substrates (Nayduch and Burrus 2016). Some studies have shown that house flies and other non-biting insects can carry multidrug-resistant bacteria (Zurek and Ghosh 2014). Antibiotic resistance in pathogens could cause major problems in preventing or stopping disease outbreaks. House flies collected from farms have even tested positive for trace amounts of African swine fever virus (Herm et al. 2020). However, they are considered not to cause measurable harm to livestock other than to affect livestock behavioral responses (Mullen and Durden 2019).

African Swine Fever Virus

African swine fever virus (ASFV) is a hemorrhagic disease that is highly contagious and creates a variety of clinical signs and lesions that resemble those of classical swine fever. ASFV is a large, enveloped DNA virus that is resistant to freezing and thawing, a wide range of pH, and

has the potential to remain infectious at room temperature for many months (Kahn 2010). It is the only member of the family Asfarviridae. All domesticated pigs and the European wild boar are affected by the disease (Bonnet et al. 2020) and all age groups of swine are equally susceptible. Warthogs and brush pigs can have the disease and be asymptomatic (Kahn 2010). ASFV is maintained in Africa by through transmission between warthogs and soft tick vectors. The spread of the virus from wildlife reservoirs to domestic pigs through the bite of an infected tick or by ingestion of infected warthog tissue. While pig blood is infectious for up to 6 weeks, the virus is not transmitted to offspring in utero (Kahn 2010). Zani et al. (2020) found that while viral DNA could be detected in decomposing wild boars, no virus isolation of ASFV was able to be obtained. Recent studies have shown that ASFV can be transmitted by two members of the Muscidae family, *Musca domestica* and *Stomoxys calcitrans* (Olesen et al. 2018; Herm et al. 2020). Another known vector of ASFV is the hog louse, *Haematopinus suis* (Olesen et al. 2018; Bonnet et al. 2020). *S. calcitrans* and *Ha. suis* are blood feeding arthropods but transmission of the virus is not strictly through blood feeding with certain evidence of the non-blood feeding fly *M. domestica* playing a role in ASFV transmission. Olesen et al. (2018) discovered that pigs can be infected through trophic transmission by ingesting stable flies, *S. calcitrans*, which have taken a blood meal from an infected pig. Since there is currently no vaccine for ASFV, prevention or euthanasian of infected animals is the only way to stop the spread of the virus (Bonnet et al. 2020).

The objectives of this study were to 1) identify the insects associated with shallow trench composting and 2) determine the vector competence of house flies for three surrogate viruses.

CHAPTER II

Arthropod Diversity and Abundance Associated with Shallow Trench Composting of Pig Carcasses

Introduction

Animal mortality management changes as new ideas and technology become available. Traditional methods like burial are becoming less common, regulated more heavily, or even banned as by the European Union (EU) due to potential of infectious agents being released into the environment (Gwyther et al. 2011; Costa and Akdeniz 2019). Troutman et al. (2014) found that with rabbit carcasses the carcasses at the center and lower layers decomposed slower rate than the ones on closer to the surface in mass burials. Some methods like incineration are still not available to most livestock producers or if available the animals have to be transported off farm (Gwyther et al., 2011). Composting is the preferred method of disposing of dead large animals but can be more difficult for mass mortality management due to the area needed for proper composting (Wilkerson 2007). New methods like shallow trench composting are being examined for the potential as the next step in mass mortality management but the insect associated with these types of systems is not well-documented.

The insects that are associated with decomposing and composted animal carcasses are

known from previous studies (Rosa et al. 2011, Dubie et al. 2017). Insect from families such as Calliphoridae, Sarcophagidae, Muscidae, Staphylinidae, Dermestidae, and Silphidae are often associated with animal carcasses (Rosa et al. 2011, Pastula and Meritt 2013). Flies in the family Muscidae have been show to reach buried remains in loose soil up to 40cm deep, while calliphorid flies only reached 10 cm deep (Gunn and Bird 2011). Both families have been shown to develop if eggs are laid pre-burial (Gunn and Bird 2011, Amendt et al. 2010). The depth and temperature have been shown the effect the time it takes for flies to colonize a carcasses. Pastula and Merritt (2013) found that by burying the carcasses 60cm instead of 30cm the flies took 2 more days to colonize it. Turner and Wiltshire (1999) found that carcasses buried in the winter were not colonized by flies until disturbed by scavengers in the spring. Once unearthed though *Calliphora vomitoria* still colonized the carcasses 105 days after burial (Turner and Wiltshire 1999). While the burial may affect the colonization of the carcasses it does not appear to have an effect on accumulated degree days (ADD). Pastula and Merritt (2013) did not find a significant difference between temperatures around carcasses buried at 30cm or 60cm and ambient temperature. Beetles in the families Histeridae, Silphidae, and Staphylinidae have been found to arrive 7 days post burial (Pastula and Merritt 2013). Silphidae are usually only associated with shallow burials (<30cm) and feed on the carrion and fly maggots (Amendt et al. 2010). Beetle families are thought to have little disease transmission potential and while bacterial transmission is known, few studies have shown mechanical transmission of viruses by flies (Khamesipour et al. 2018; Graczyk et al. 1999; Fasanella et al. 2010). However, recent studies have shown that two members of the muscid family, *Musca domestica* and *Stomoxy calcitrans*, are potential vectors of African swine fever virus (ASFV) (Olesen et al. 2018; Herm et al. 2020). The objective of this study was to identify insects associated with shallow trench composting.

Materials and Methods

Traps

Two trap types were used for this study; malaise and pitfall. The malaise traps were H shaped traps from Bio Quip and established according to the manufacturer's directions (Figure 1). The preliminary / control malaise trap was set with openings running North and South while the animal mortality trench malaise traps were opened East and West. The pitfall trap sets were constructed by using 1000mL paint cups with lids, garden edging, and garden stakes (Fig. 2). The garden edging was cut into 60cm lengths and staked in the ground with two garden stakes. Each trap set had one central cup and three garden edging rays with another cup at the end of each ray. The lids of the paint cups were suspended over the cups with two garden stakes to help prevent rain from entering and to reduce evaporation. Posthole diggers and garden trowels were used to dig the holes for the cups. The lip of the cups were placed at ground level. Prestone® lowtox antifreeze was added to the pitfall traps as a killing and preserving agent.

Preliminary traps were set out two weeks before the animal mortality trenches were dug. These traps consisted of one malaise and three sets of pitfall traps. The purpose of these traps were to give a baseline to compare to the traps placed near the trenches since space was limited. The traps were collected every two to three days and then became the control traps once the first set of pigs were placed in the animal mortality trenches. The traps over the trenches were placed the day after animal placement due to time restrictions. The pigs were placed in two sets, one week apart. Around each trench 10 sets of pitfall trap sets were placed, five on each side, and one malaise trap. The final trap layout is shown in Figure 3. Traps were collected every two to three days for the first month and then time between trap collection increases with a decrease in specimens collected. The contents of the traps were placed in 70% ethanol for later identification. Traps were repaired and reset as needed.

Figure 1: Malaise trap set up for the Preliminary site.

Figure 2: Pitfall trap set. A total of 23 arrays were established for this study.

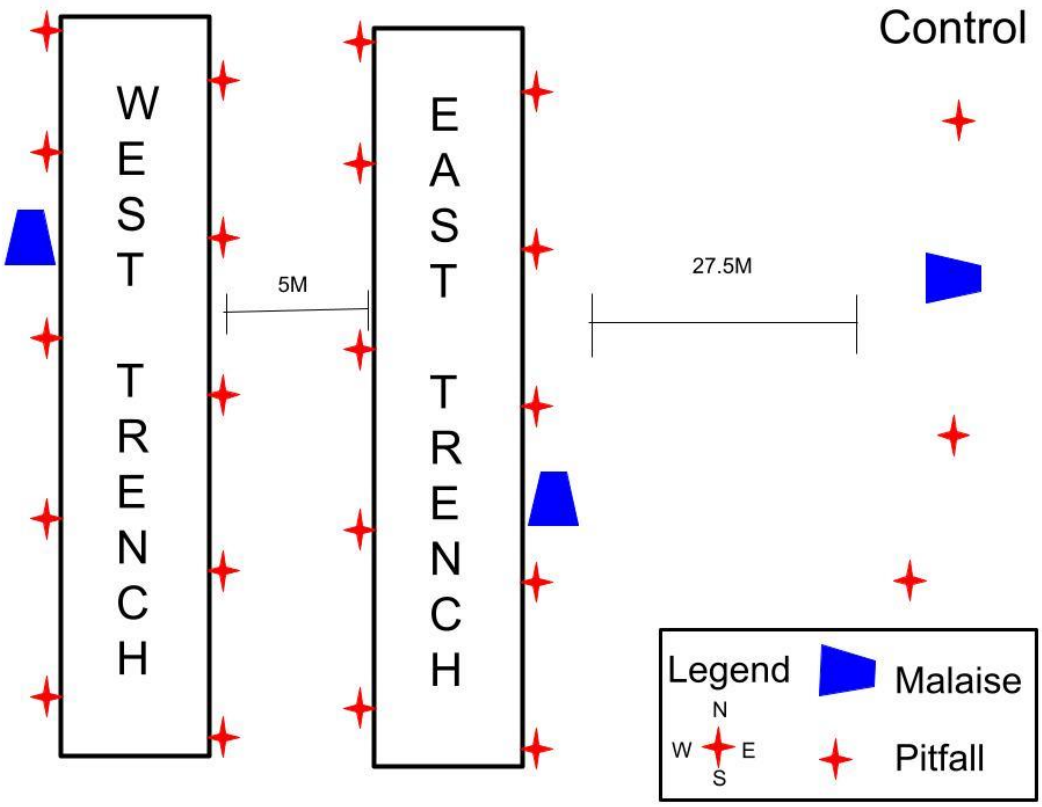

Figure 3: Map of the field site and trap layout.

Animal Mortality Trenches

One hundred culled sows approximately 204kg each were obtained from swine production houses in western Oklahoma, euthanized, inoculated with a swinepox virus, and placed in two trench over a period of two weeks with half placed each week. The trenches were 50 M long, 2 M wide, 0.6M deep, and filled with 0.3 M of wood chips. Sows were placed in groups of 5 with a 15 cm gap between the groups to help separate the groups during femur collection. After inoculation and before burial the abdomens of each pig was lanced to prevent gas buildup. Hobo Data Loggers® were placed in the abdomen and at the pig-soil interface on pigs 12, 24, 36, and 48. The data loggers collected the temperature once every hour for the duration of the trial. The trenches then had 4.8 m horse panels placed around them to discourage scavengers (Fig. 4).

Post burial 5 pig carcasses per trench were dug up and the inoculated femurs were removed at weeks 1, 2, 3 and months 1, 2, 6, and 12. Insects were collected at the time of femur retrieval from the holes dug to retrieve the femurs.

Figure 4: Animal mortality trench after animal and trap placement.

Results

Animal Mortality Trenches Temperature

Mesonet data collected from the Lake Carl Blackwell station shows a steady decline in the temperature after animal placement (Fig. 5). The temperature of the pigs in the trenches remained above 30^{o}C then started to decrease at a faster rate after the 22nd of September (Fig. 6 and 7). The west trench temperature data has is missing some dates in the beginning because

during burial the loggers were unplugged. This data was removed to better show the trend in the temperature. The graphs focuses on one month post burial for the degree hour model. This timeframe includes all expected emergence dates (Table 1), the virus viability time (7 days post burial), and the peaks in forensically important flies.

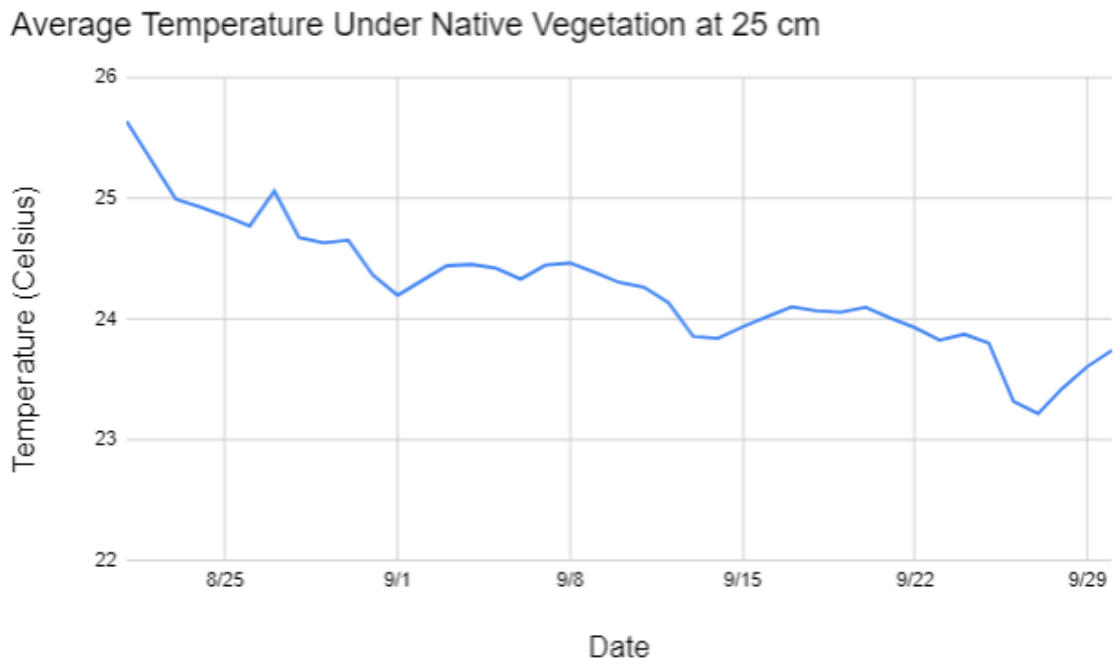

Figure 5: Mesonet temperature collected 25cm underground from Lake Carl Blackwell station aprroximantily 100m from field site.

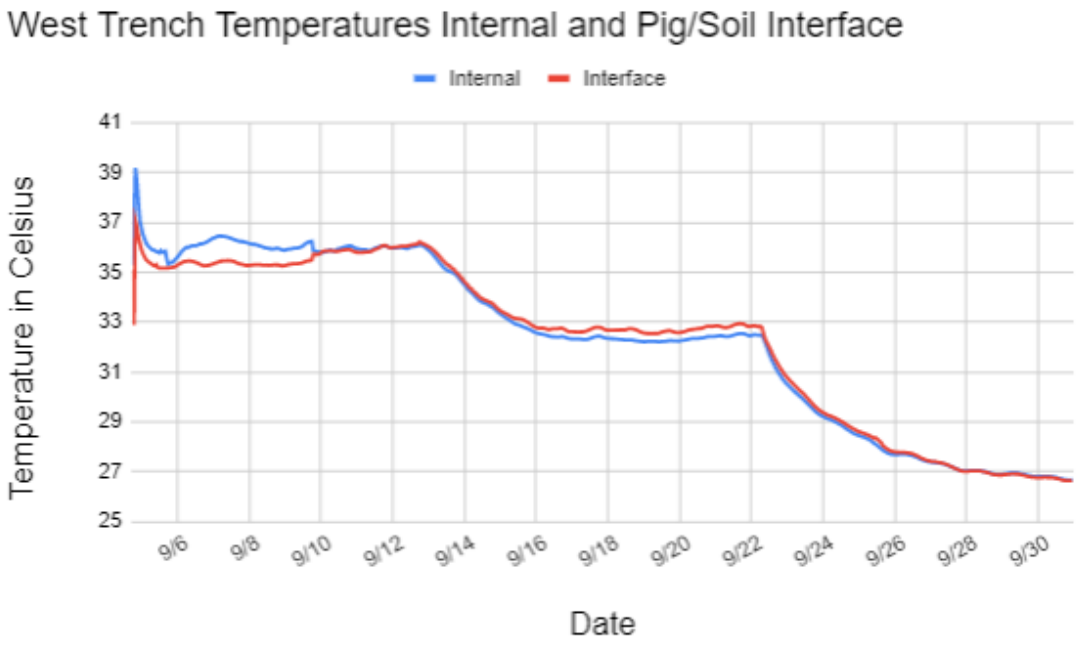

Figure 6: Temperature of West trench through September 30th

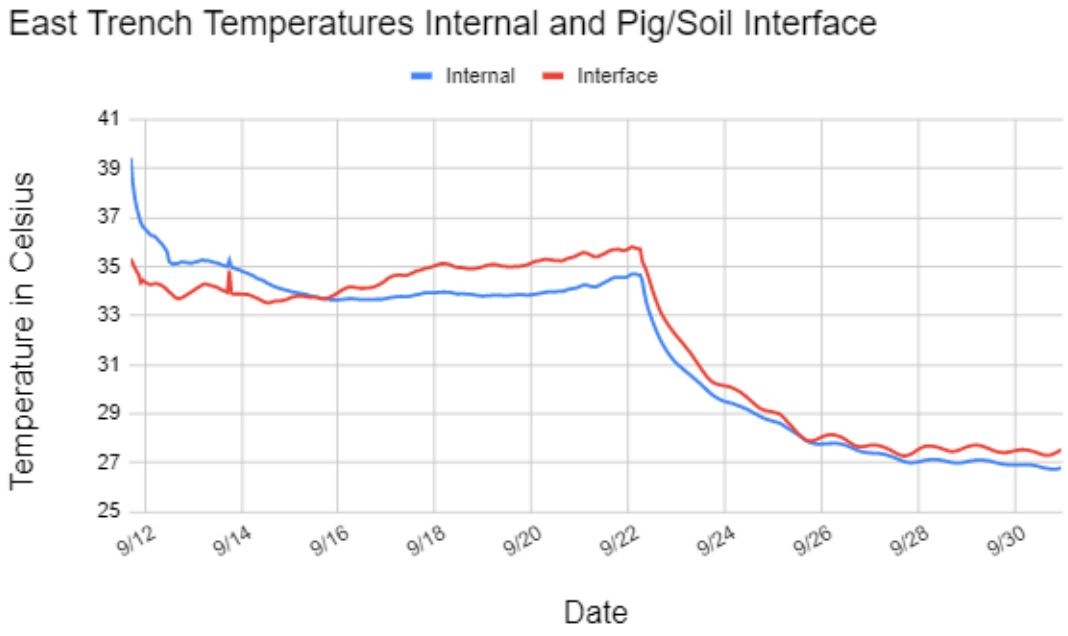

Figure 7: Temperature of the East trench through September 30th.

Fly Degree Hour Model

A predictive degree hour model (Table 1) was developed based on previous work done for specific species for common calliphorid flies and the two proven vectors of African swine fever virus, *Musca domestica* and *Stomoxys calcitrans*. The predictive model demonstrated peaks of forensically important files on September 12th-13th and September 19th-20th. Specimens within the Calliphoridae family had the highest abundance of forensically important fly families with peaks in adults collected on September 16th, 22nd, and 27th (Fig. 8 and Fig. 9). Specimens in the families Muscidae, Sarcophagidae, and Phoridae also demonstrated an increase in abundance on the 16th of September. The highest peak for Muscidae, Calliphoridae, and Phoridae was on September 27th. Sarcophagidae highest peak was on the 25th of September. The predictive model shows an expected increase in fly activity 8-9 days post burial, which is supported by the timeline of forensically important flies collected. Burial date were 9/4 and 9/11 respectively for the west and east trenches.

Table 1: Predictive emergence of adult Muscidae and Calliphoridae fly species based on degree hour models.

Species	Lower Developmental Threshold	Accumulated Degree Hours Needed (ADH)	Date ADH was Achieved Per Trench	
			West	East
Stomoxys calcitrans[A]	10°C	5832	9/13	9/20
Musca domestica[A]	10°C	5328	9/13	9/20
Phormia regina[B]	10°C	4976	9/12	9/19
Lucilia sericata[C]	10°C	5069	9/12	9/19
Cynomya cadaverina[C]	6°C	5511	9/13	9/20
Cochliomyia macellaria[D]	10°	4698	9/12	9/19

[A]= Lysyk 1993
[B]= Nabity et al. 2006
[C]= Wang et al. 2016
[D]= Boatright and Tomberlin 2010

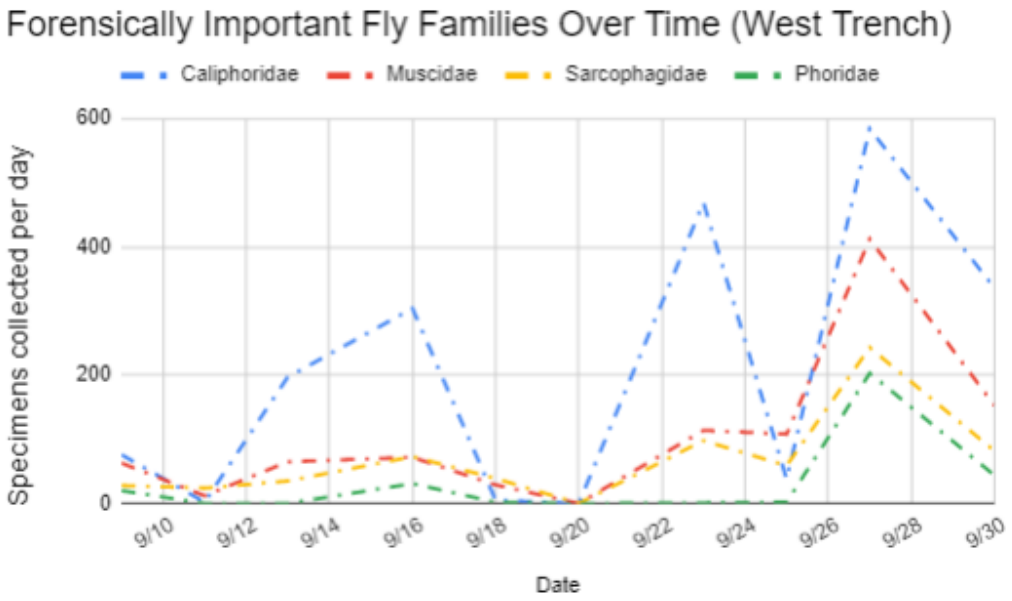

Figure 8: Number of forensically important flies collected per day from the west animal mortality trench near Stillwater, OK in 2019.

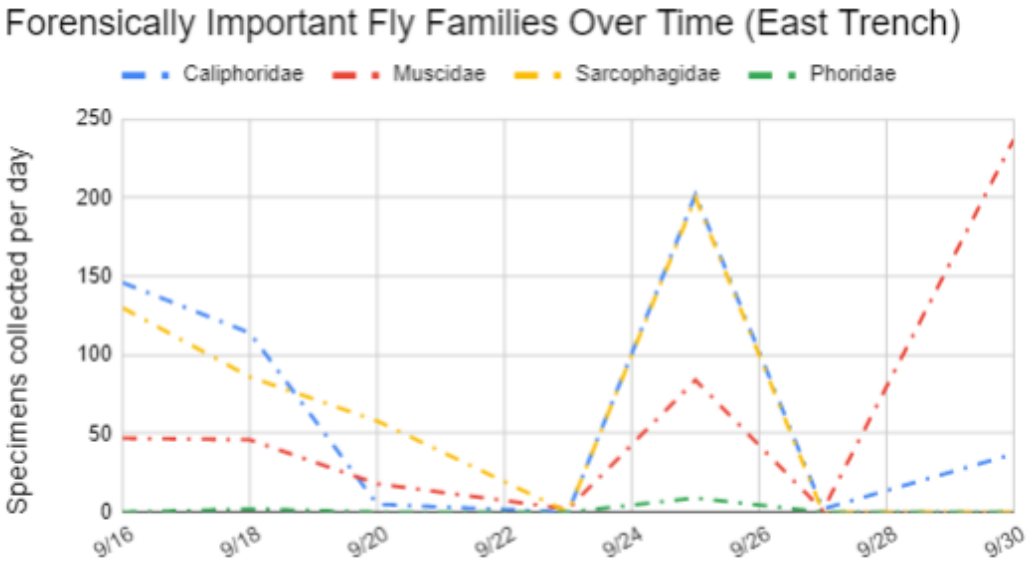

Figure 9: Number of forensically important flies collected per day from the east animal mortality trench near Stillwater, OK in 2019.

Diversity and Abundance

A total of 28,575 specimens were identified from the field site. The total specimens in pitfall traps total 5,845 specimens, with the primary orders being Coleoptera, Hymenoptera, and Orthoptera. The preliminary, control, and animal mortality trenches had 665, 1,843, and 3,337 total specimens, respectfully. Hymenoptera and Orthoptera specimens were the dominant taxa collected in the preliminary (Fig. 10) and control (Fig. 11) but in the animal mortality trench pitfall traps almost half of all specimens collected were in the order Coleoptera (Fig. 12). There were significantly more Coleoptera ($X^2(1, N=1722) = 541.80$, $p>0.05$) and Orthoptera ($X^2(1, N=1010) = 21.83$, $p>0.05$) in the animal mortality trenches than the control site pitfall traps.

A total of 22,730 specimens were identified from the malaise traps with the dominant specimens representing the orders Diptera (16,148), Hymenoptera (3,496), and Hemiptera (1,977). Dipteran specimens remained the large group across the malaise trap accounting for 71% of the total specimens caught in the malaise traps. The percentage of Hymenoptera specimens increased from the preliminary site (Fig. 13) to the control site (Fig. 14) with the animal mortality trenches exhibiting the highest percentage of Hymenopteran (Fig. 15) while Diptera decreased in total percentage from the malaise traps from 82% to 71% of total specimens. Even though the total of percentage collected decreased the number of dipteran specimens collected rose from 427 in the preliminary malaise traps to 16,148 in the animal mortality trenches. There were significantly more Diptera ($X^2(1, N=20,658) = 3,278.22$, $p>0.05$), Hemiptera ($X^2(1, N=4152) = 971.29$, $p>0.05$) and Hymenoptera ($X^2(1, N=2307) = 587.91$, $p>0.05$) in the animal mortality trenches than the control site pitfall traps

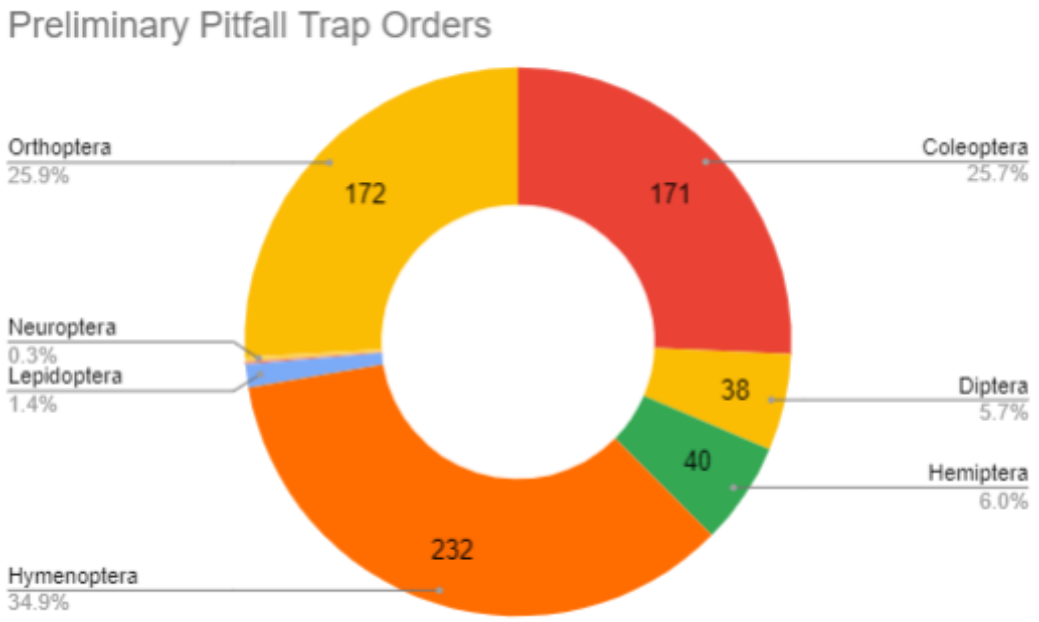

Figure 10: Percentage and number of specimens associated with certain insect orders collected in preliminary pitfall traps.

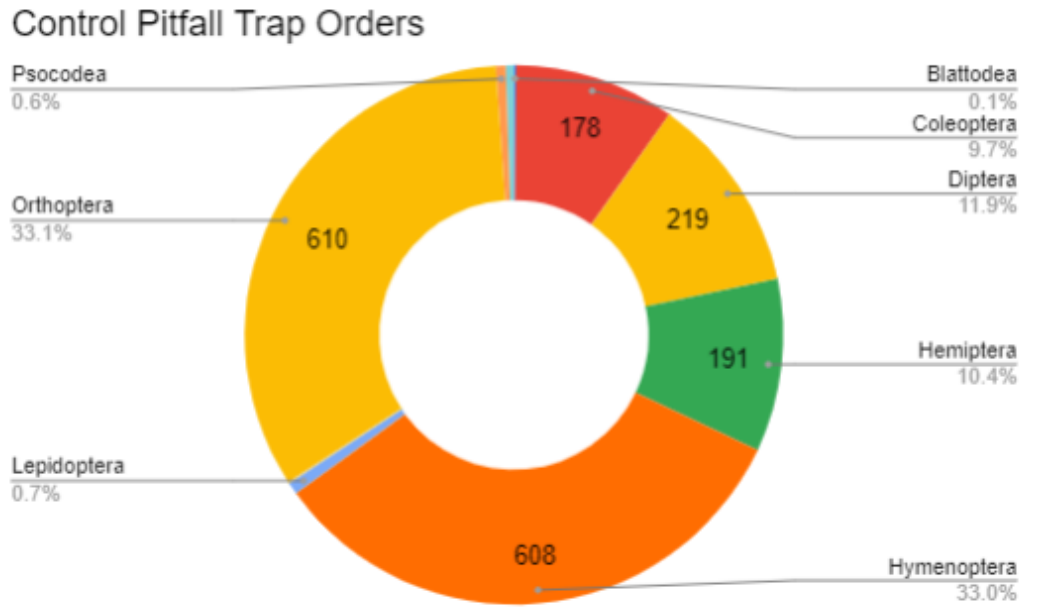

Figure 11: Percentage and number of specimens associated with certain insect orders collected in control pitfall traps.

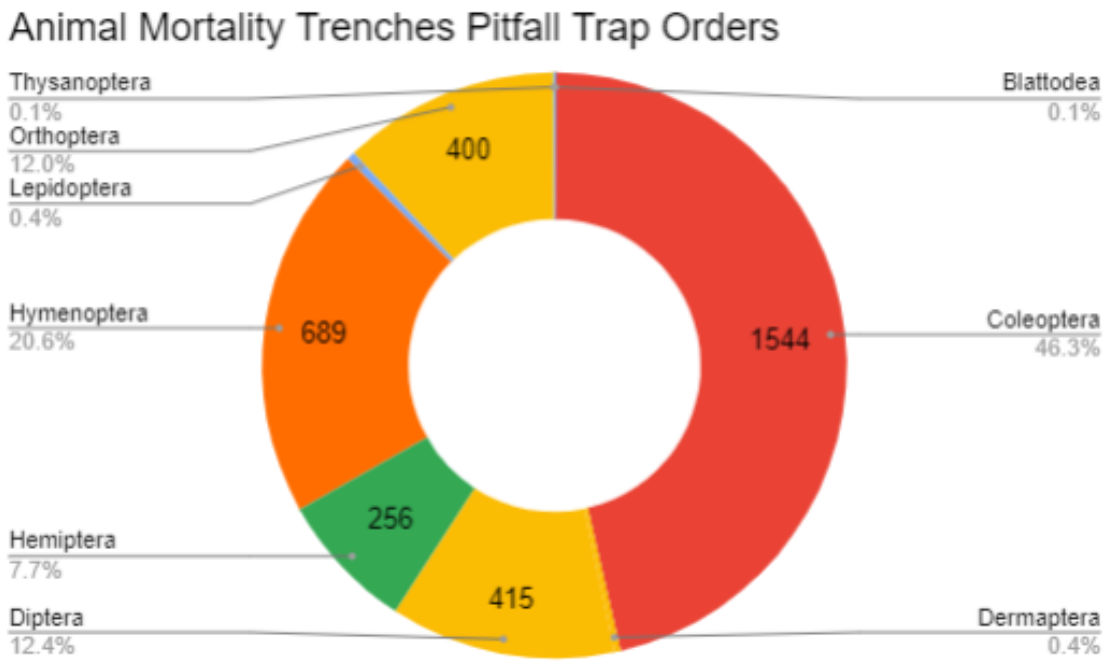

Figure 12: Percentage and number of specimens associated with certain insect orders collected in pitfall traps around the animal mortality trenches.

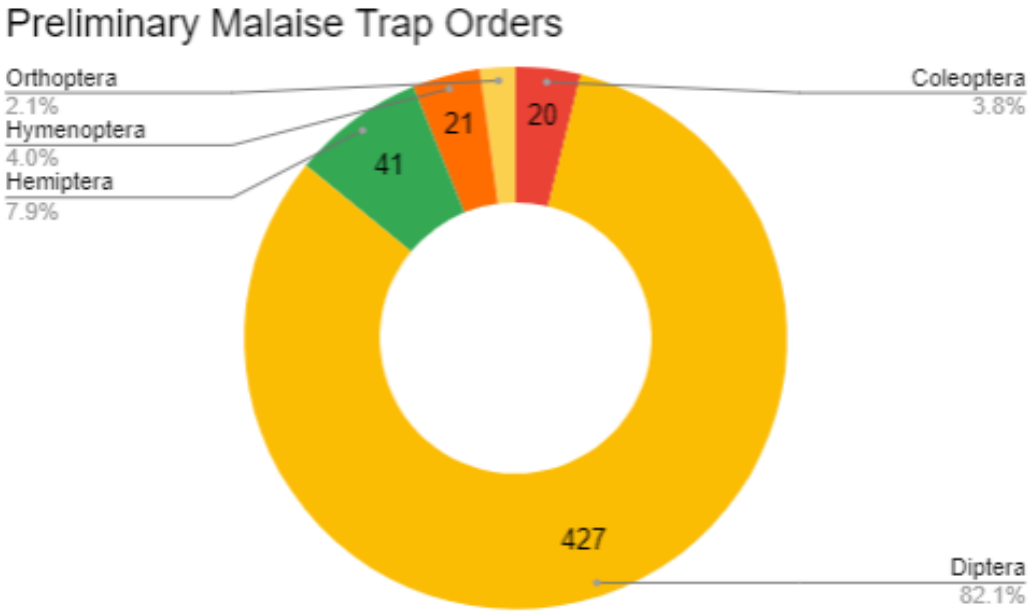

Figure 13: Percentage and number of specimens associated with certain insect orders collected in preliminary Malaise traps.

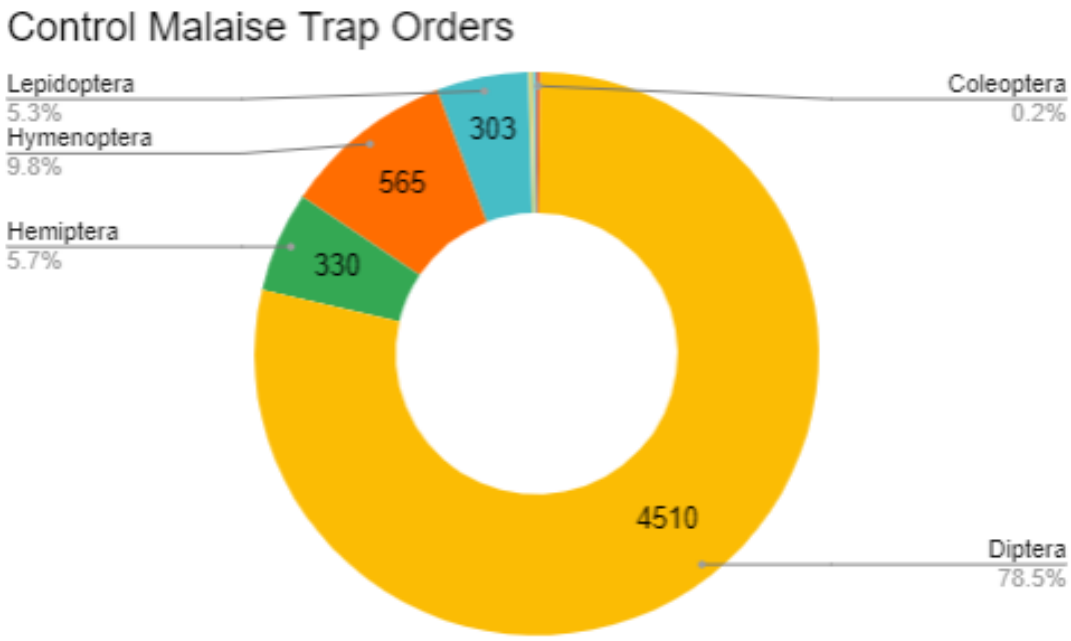

Figure 14: Percentage and number of specimens associated with certain insect orders collected in control Malaise traps.

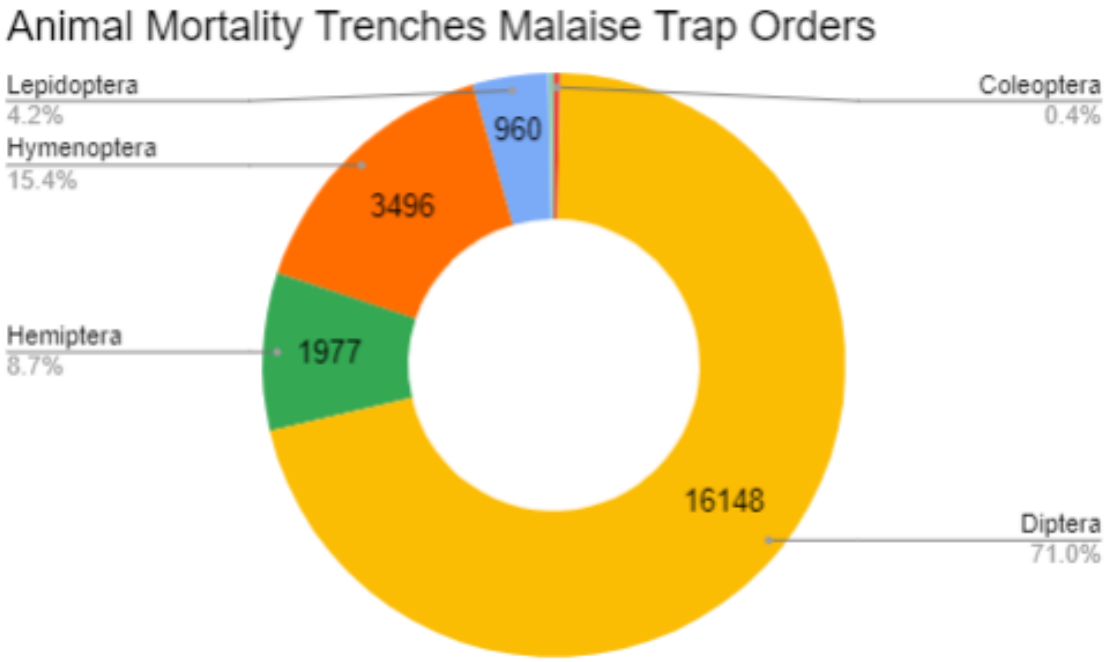

Figure 15: Percentage and number of specimens associated with certain insect orders collected in Malaise traps around the animal mortality trenches.

There was a total of 1,893 beetles (Coleoptera) (Table 2) and 672 flies (Diptera) (Table 3) collected in pitfall traps. Forensically important beetle families totaled 1,084 specimens. A total of 480 Scarabaeidae, 320 Silphidae, 185 Staphylinidae, and 7 Trogidae in the pitfall traps located near the animal mortality trenches. Thirty-one Scarabaeidae and 17 Staphylinidae were collected in the control traps. Thirty-nine Scarabaeidae and 4 Staphylinidae were caught in the preliminary traps. Scarabaeidae and Staphylinidae with not significantly different between animal mortality trenches and preliminary (Scarabaeidae, $X^2(1, N=519) = 1.6$, $p>0.05$; Staphylinidae, $X^2(1, N=202) = 0.04$, $p>0.05$) or control (Scarabaeidae, $X^2(1, N=511) = 2.7$, $p>0.05$; Staphylinidae, $X^2(1, N=189) = 3.0$, $p>0.05$) pitfall traps. Animal mortality trench pitfall traps collected significantly more Silphidae than the control ($X^2(1, N=320) = 8.0$, $p<0.05$) and preliminary ($X^2(1, N=320) = 8.0$, $p<0.05$) pitfall traps. A total of 285 fly specimens that are considered forensically important were caught during the preliminary sampling periods. A total of 39, 7, and 0 fly Calliphoridae specimen(s) were caught in pitfall traps near the animal mortality trenches, control and preliminary pitfall traps, respectively. The Calliphoridae specimens collected in the pitfall traps were not significantly different when comparing the animal mortality trenches to the control ($X^2(1, N=46) = 0.01$, $p>0.05$) or the preliminary ($X^2(1, N=39) = 1.0$, $p>0.05$). A total of 13, 12, and 1 fly specimen(s) in the family Sarcophagidae and a total of 16, 14, and 0 fly specimen(s) in the family Muscidae were caught in the animal mortality trenches, control and preliminary pitfall traps, respectively. The Sarcophagidae specimens collected in the pitfall traps were not significantly different when comparing the animal mortality trenches to the control ($X^2(1, N=25) = 1.3$, $p>0.05$) or the preliminary ($X^2(1, N=14) = 0.1$, $p>0.05$).The Muscidae specimens collected in the pitfall traps were not significantly different when comparing the animal mortality trenches to the control ($X^2(1, N=30) = 1.4$, $p>0.05$) or the preliminary ($X^2(1, N=16) = 0.4$, $p>0.05$). A total of 148, 28, and 7 fly specimens within the Phoridae family were caught in the animal mortality trenches, control, and preliminary, respectively. The Phoridae specimens collected in the pitfall

traps were not significantly different when comparing the animal mortality trenches to the control ($X^2(1, N=176) = 0.1$, $p>0.05$) or the preliminary ($X^2(1, N=155) = 1.3$, $p>0.05$).

A total of 16,148 flies (Diptera) (Table 3) and 79 beetles (Coleoptera) (Table 2) were caught in both pitfall and malaise traps. Of the 16,148 flies, 9,155 were from forensically important families with the animal mortality trench malaise traps containing 2,568 Calliphoridae, 1,542 Sarcophagidae, 2,169 Muscidae, and 202 Phoridae. The control traps were significantly different than the animal mortality trenches containing 322 Calliphoridae ($X^2(1, N=1,606) = 288.1$, $p<0.05$), 1,452 Sarcophagidae ($X^2(1, N=2,994) = 104.3$, $p<0.05$), 657 Muscidae ($X^2(1, N=2,826) = 52.4$, $p<0.05$), and 31 Phoridae ($X^2(1, N=233) = 18.6$, $p<0.05$). The preliminary traps were also significantly different than the animal mortality trenches containing 4 Calliphoridae ($X^2(1, N=2572) = 636.0$, $p<0.05$), 172 Sarcophagidae ($X^2(1, N=1,714) = 190.2$, $p<0.05$), 36 Muscidae ($X^2(1, N=2,205) = 490.6$, $p<0.05$), and 0 Phoridae ($X^2(1, N=202) = 50.5$, $p<0.05$). No beetles from forensically important families were found in the malaise traps during the preliminary sites.

Silphidae were the only coleopteran family found in traps around the animal mortality trenches and not at any other sampling area. This is to be expected since these beetles are commonly associated with carrion (Dubie and Talley 2017, Rosa et al. 2011). There was an increase in specimens in the dipteran families of Calliphoridae, Muscidae, and Sarcophagidae, which are all commonly associated with decomposing animals. Calliphorid flies was the most abundant flies collected in the animal mortality trenches.

Table 2: Total number of Coleopteran specimens collected per family from each sampling area (trench = animal mortality trenches; control = located away from the trenches; prelim = preliminary site before disturbance and animal placement).

Family	Trench Pitfall	Trench Malaise	Control Pitfall	Control Malaise	Prelim Pitfall	Prelim Malaise
Annobidae	1	0	15	0	0	0
Anthibidae	11	0	1	0	0	0
Bostrichidae	34	0	1	0	2	0
Buprestidae	6	1	1	0	0	0
Cantharidae	3	5	2	0	0	0
Carabidae	129	2	12	4	68*	0
Cerambycidae	212	7	41	4	0*	18*
Chrysomelidae	45	10	7	2	17	1
Coccinellidae	0	0	0	0	0	1
Curculionidae	59	8	17	0	10	0
Dermestidae	0	1	0	0	0	0
Elateridae	1	0	7	0	5	0
Erotylidae	2	1	1	0	0	0
Eucinetidae	0	0	1	0	0	0
Histeridae	3	0	2	0	0	0
Lampyridae	1	2	0	1	0	0
Meloidae	18	2	7	0	25	0
Mordellidae	4	2	8	0	0	0
Phalacridae	0	7	0	0	0	0
Ptilodactylidae	0	0	2	0	0	0
Scarabaeidae	480	0	31	0	39	0
Silphidae	320	0	0*	0	0*	0
Staphylinidae	185	0	17	0	4	0
Tenebrionidae	1	0	0	0	0	0
Troogidae	5	0	1	0	2	0
Unknown	24	0	3	0	0	0

* $p<0.05$, chi squared 3.841, df=1. Significance was compared between animal mortality trenches and control or animal mortality trenches and preliminary for families with over 200 specimens collected or forensically important.

Table 3: Dipeteran family totals from each sampling area (trench = animal mortality trenches; control = located away from the trenches; prelim = preliminary site before disturbance and animal placement).

Family	Trench Pitfall	Trench Malaise	Control Pitfall	Control Malaise	Prelim Pitfall	Prelim Malaise
Anthomyiidae	0	805	8	50*	1	0*
Asilidae	0	2	0	4	0	0
Bombilidae	0	2	0		0	0
Calliphoridae	39	2568	7	322*	0	4*
Chironomidae	0	1	0	0	0	0
Choloropidae	0	1	0	0	0	0
Culicidae	87	1836	86*	1195*	21	1*
Dolichopodidae	3	1054	43*	319*	4	105*
Drosophilidae	0	0	4	1	2	0
Empididae	0	20	0	0	0	7
Ephydridae	0	20	3	22	0	0
Lauxaniidae	0	0	0	3	0	3
Muscidae	16	2169	14	657*	0	36*
Phoridae	148	202	28	31*	7	0*
Sarcophagidae	13	1542	12	1452*	1	172*
Scathophagidae	0	23	1		0	0
Simullidae	0	5	2	10	0	0
Stratiomyidae	0	2	0	0	0	0
Syrphidae	0	129	3	48	0	2
Tabanidae	0	16	1	32	0	6
Tachinidae	0	40	0	43	0	2
Tephritidae	0	12	0	17	0	48
Tipulidae	0	89	1	42	0	5
Ulidiidae	1	387	2	98*	2	0*
Unknown	108	286	4	164	0	36

* $p<0.05$, chi squared 3.841, df=1. Significance was compared between animal mortality trenches and control or animal mortality trenches and preliminary for families with over 200 specimens collected or forensically important.

Forensically important insects were identified to lowest possible taxa listed on Table 4. The majority were only collected after placement of animals had occurred. There was a significant increase in the beetle genera and species were identified around the animal mortality

trenches compared to the control site (X^2 (1,N=25)=4, p<0.05) while the flies are not significantly different between the trenches and the control site (X^2 (1,N=24)=0.13, p>0.05). *Musca domestica*, a proven vector of African swine fever virus, was found in both the trenches and control sites. Although this common fly was caught in both the animal mortality trenches and control site this fly caught in such low numbers there is no observable trend to determine if this was statically different or was associated with the animal mortality trenches (X^2 (1,N=5)=0.1, p>0.05). *Necrodes surinamensis* and *Cochliomyia macellaria* were both collected around the animal mortality trenches but not the other sites.

Table 4: List of genera and species taxa identified. (+) = is at least one specimens of that species was found in either a pitfall or a malaise trap for that site.

Coleoptera	**Preliminary**	**Control**	**Mortality Trenches**
Staphylinidae			
Aleocharinae			
Homalota spp.			+
Phanerota spp.		+	
Dasycerinae			+
Leptotyphlinae			+
Habrocerinae ***Habrocerus spp.***			+
Micropepinae			+
Oxyporinae ***Oxypus spp.***		+	+
Oxytelinae			+
Paederinae ***Homaeotarus spp.***			+
Scaphidiinae ***Cyparium spp.***			+
Staphylininae			
Ocypus spp.			+
Platydracus spp.		+	+
Scarabaeidae			
Aphodiinae ***Ataenius A. confertus***		+	+
Cetoniinae ***Euphoria E. sepulcralis***	+	+	+
Malancanthon			+
Aphodiinae ***Aphodius spp.***	+	+	+

Melolontine ***Hoplia spp.***		+	+
Omalinae			
Occiephilinus spp.			+
Haida spp.			+
Vellica spp.			+
Scarabaeinae			
Ateuchus ***A. hiseroides***			+
Phanaeus spp.			+
Onthophagus ***O. pennsylvanicus***			+
Silphidae ***Necrodes*** ***N. surinamensis***			+
Trogidae ***Omorgus spp.***	+	+	+
Diptera			
Muscidae			
Atherigona ***A. reversura***	+	+	+
Caricea spp.			+
Eudasyphora ***E. cyanella***		+	+
Fannia spp.		+	
Haematobia irritans			+
Musca ***M. domestica***		+	+
Neodexiopsis ***N. ovata***	+	+	+
Phaonia spp.		+	+
Stomoxys ***S. calcitrans***		+	
Sarcophagidae			
Boettcheria spp.		+	+
Ravinia spp.	+	+	+
R. stimulans		+	
Sarcophaga spp.		+	+
Calliphoridae			
Cochliomyia		+	+
C. macellaria			+
Cynomya spp.			+
Lucilia			
L. silvarum		+	
L. coeruleiviridia		+	
L. elongata			+
L. eximia			+
L. sericata	+	+	+
Pollenia			
P. griseotomentosa			+

P. pediculata			+
P. rudis			+
Phoridae			
Dohriniphora spp.		+	
Stichilus spp.		+	+

The percent change in certain forensically important fly families such as Muscidae, Calliphoridae, and Phoridae was larger than the percent change in Sarcophagidae in the control malaise trap compared to the animal mortality trench malaise traps (Table 5). The percent change in Calliphorid specimens between the animal mortality trenches and the preliminary malaise traps was the largest at 32,000%. The percent change in important dipteran families suggests that animal placement into a mortality management area will increase fly abundance especially when comparing the preliminary and control sites to the trench sites.

Table 5: Average number of Calliphorids, Sarcophagids, Muscids and Phorids per malaise trap and percent change compared either from the preliminary sites or control sites to the animal mortality trenches.

	Calliphoridae	Sarcophagidae	Muscidae	Phoridae
Trench Malaise	1284	771	1084.5	101
Control Malaise	332	1452	657	31
Prelim Malaise	4	172	36	0
%change Malaise Control/Trench	299%	*46%	65%	225%
%change Malaise Prelim/Trench	32,000%	348%	291%	N/A

Simpson's index (S), Shannon-Wiener index (SW), evenness (E), and richness (R) were calculated for each of the sites. Order diversity was the highest in the animal mortality trenches (0.395 S and 1.256 SW) followed by the control site (0.426 S and 1.232 SW,) then the preliminary site (0.618 S and 0.743 SW) (Table 6). The dipteran families followed the same pattern with animal mortality trenches (0.147 S and 2.119 SW), control (0.202 S and 1.937 SW), and preliminary (0.219 S and 1.857 SW). The control site was the most diverse in with coleopteran families (0.122 S and 2.416 SW) then animal mortality trenches (0.175 S and 2.064

SW) and preliminary (0.204 S and 1.857 SW). The animal mortality trenches had the lowest evenness (.657 E) for coleopteran families indicating higher presence of coleopteran taxa. The animal mortality trenches the highest number of taxa across all three categories shown by the richness index (11 R in orders, 23R for both family groups). The animal mortality trenches had 5 more orders, 12 more coleopteran families and 5 more dipteran families that the preliminary site. The animal mortality trenches also had 2 more coleopteran families and 3 more dipteran families than the control site with the same number of orders.

Table 6: Diversity and abundance indexes for orders, Coleoptera families, and Diptera families from each trap group.

	Preliminary	**Control**	**Animal Mortality Trenches**
Orders			
Simpson's	**0.681**	**0.426**	**0.395**
Shannon-Wiener	**0.743**	**1.232**	**1.256**
Evenness	**0.414**	**0.514**	**0.524**
Richness	**6**	**11**	**11**
Coleoptera Families			
Simpson's	**0.204**	**0.122**	**0.175**
Shannon-Wiener	**1.857**	**2.416**	**2.064**
Evenness	**0.774**	**0.794**	**0.657**
Richness	**11**	**21**	**23**
Diptera Families			
Simpson's	**0.219**	**0.202**	**0.147**
Shannon-Wiener	**1.894**	**1.937**	**2.119**
Evenness	**0.669**	**0.646**	**0.676**
Richness	**17**	**20**	**23**

None of the insects collected while femur samples were collected tested positive for Swinepox Virus. These insects were not counted in the total number of insects or in any calculations.

Discussion

This study has shown that shallow trench composting attracts similar entomological fauna as other methods of mass animal mortality management (Rosa et al. 2011). Both this study and Rosa et al. (2011) found Diptera to be the most dominant order at 56% and 80%, respectfully,

and Sarcophagidae, Muscidae, and Calliphoridae to be the most dominant fly families. The number of individuals within the Calliphoridae family did not consist of as large of a portion as in some previous studies, 18% compared to 49% of total flies collected (Dubie and Talley 2017). This agrees with previous studies that stated Calliphoridae are not found past 10cm deep unless place on the carcasses pre-burial (Amendt et al. 2010, Gunn and Bird 2011). Calliphoridae, Muscidae, and Culicidae were the three dipeteran families with the largest representation in the traps that were around the animal mortality trenches. Dubie et al. (2017) also found Calliphorid and Muscid flies as two of the most common families in terms of specimens collected but did not report any specimens in the family of Culicidae. Standing water was present at the trap sites which likely explains the large number of mosquitoes (Fig. 16). Another possibility for the presents of mosquitoes is that the decomposition generates CO_2 that keeps them in the same area. The species found by Dubie and Talley (2017) were similar with a few noticeable differences. Dubie and Talley (2017) identified more taxa in the families Sarcophagidae and Calliphoridae but less in the Muscidae family. Both studies commonly found *Musca domestica* a proven vector of African Swine Fever Virus (Dubie and Talley 2017). The only species of Silphidae found in our study was *Necrodes surinamensis* while the other study found *N. surinamensis, Necrophilia americana,* and other unidentified species (Dubie and Talley 2017).

Silphidae were only caught in the traps around the animal mortality trenches and were observed crawling in and out of the dirt that covered the animal mortality trenches. This study was conducted in a field setting while Dubie and Talley (2017) conducted their study close to a feedlot. This change in habitat could account for the diversity difference. *Phlionthus spp.* a member of the subfamily Staphylininae was mainly found by Gaudry et al. (2006) at 10cm and 30 cm deep. Similarly this study found more beetles in this family and subfamily, though not significantly different, in the animal mortality trenches compared to the preliminary and control site. Beetles in the family Histeridae were not collected in this study. Previous studies have

collected these beetles (Amendt et al. 2010, Pastula and Merritt 2013, Dubie and Talley 2017). These beetles are usually collected on ~7 days after burial (Pastula and Merritt 2013). One thing to note is that most other studies did not mention the method of collection. The two studies that did were Dubie and Talley (2017) and Pastula and Merritt (2013) and they used pitfall and hand collection, respectfully. Difference in collection type could explain some of the difference in insect fauna. Parasitic Hymenoptera have been found in burial site up to one meter deep (Amendt et al. 2010). Parasitic hymenoptera could explain the significant increase in the order between animal mortality trenches and the control site. The animal mortality trenches found Due to the time it took to inoculate the femurs of the pigs, the pigs were exposed to flies between 9 to 11 hours before burial. This allowed for flies to oviposit their eggs on the carcasses which could have resulted in the mass emergence observed (Fig. 17). It is unclear if that was the sole reason for the mass emergence. Muscid flies have been shown to travel 10cm down in compacted soil and 40cm in loose soil (Gunn and Bird 2011). Other possible explanations are the wooden stakes used to mark the location for certain groups of pigs possible created an opening for the flies (Fig. 17) to emerge. Another route for flies to emerge or gain access to the buried pigs was if the zip ties used to locate the femurs of the swine were exposed and could allow flies to oviposit eggs onto the pig carcasses. Calliphorid flies have been known to oviposit on buried carcasses 105 day old after they have been exposed by scavengers (Turner and Wiltshire 1999). When collecting femurs we exposed the pigs for approximately 1 hour. This could have attracted more flies and allowed them to oviposit more eggs on the carcasses. This could also explain the additional peak in the collected flies' timeline. However combined with the degree hour model and the collection timeline, it is possible to conclude that the eggs were laid on the day of burial. Viable virus inoculated into the femurs of the pigs was recovered 7 days post burial. The six species of flies in the degree hour model had expected adult emergence after 8 or 9 days. This is enough time to reach third instar larvae for the flies with viable virus in the animal mortality trenches. Pastula and Merritt (2013) found that burying the carcasses at 30cm or 60cm does not significantly affect

the accumulated degree days when compared to the ambient temperature. This study showed a ~6 day increase in the expected emergence date when comparing the Mesonet data to the Hobo® data loggers' data. One factor that need to be considered when comparing Mesonet to the Hobo® is the Mesonet only had a daily average and the Hobo® had an hourly average. Another point is that Pastula and Merritt (2013) was examining burials and this study was shallow trench composting. The composting could retain or produce heat. A potential issue that highlights the importance of completely covering of buried carcasses were some areas of the pigs were exposed after the burial process (Fig. 19) which allowed certain Calliphorid flies to colonize those exposed areas. These areas were completely covered after a thorough inspection of both animal mortality trenches. Both Muscid and Calliphorid flies have been shown to lay eggs on the surface of the soil so the larvae can crawl down to the carcasses (Amendt et al. 2010, Gunn and Bird 2011).

Figure 16: Water standing between the two animal mortality trenches.

Figure 17: Dipteran larve emerging from one of animal motrality trenches.

Figure 18: Wooden stake used to mark were certain pig groups were located within one of the animal mortality trenches with flies resting on it.

Figure 19: Exposed pig snout with Calliphoridae flies crawling over it from an area of the animal mortality trenches that were not completely covered.

CHAPTER III

Virus Viability in house flies (*Musca domestica*) Exposed to Swinepox Virus, Senecavirus A, and Bovine Viral Diarrhea Virus

Introduction

Vector-borne diseases are known from all over the world and across many species. Some microorganisms have no effect on their host while others cause symptoms and even death. Large disease outbreaks in animal facilities can result from viral infection and cause the need to depopulate a large number of animals in a short time period. In these events, the carcasses of the animals can be exposed to the environment and insects for an extended period of time. The most common and well-studied vectors are blood feeding arthropods such as ticks, lice, fleas, mosquitoes, and biting flies.

Flies are usually the first to arrive at carcasses (Rosa et al. 2011). In chapter II, Dubie and Talley (2017), and Watson and Carlton (2005) Calliphoridae was the most common filth fly family in animal mortality areas. Filth flies are associated with bacterial pathogens and some studies have shown flies in the family Muscidae to transmit viruses (Oelsen et al. 2018; Khamesipour et al. 2018; Nayduch and Burrus 2017). Filth flies usually transport pathogens through contaminated mouthparts, legs, etc. or through the regurgitation of previously ingested food. Mechanical transmission like this is considered less efficient than biological transmission

by blood feeding insect (Mullen and Duren 2019).

House flies have been shown to transmit human pathogen like *Eschericha coli, Salmonella* spp., *Shigella* spp., and *Staphylococcus* spp. (Nayduch and Burrus 2017, Chaiwong et al. 2014, Boulesteix et al. 2005). They are known to also move bacteria around feedlot and surrounding area mechanically, but are thought to have little adverse effect on animal growth and development (Kahn 2010). However, knowledge about viability of viruses in exposed flies is restricted. African swine fever viral DNA has been found on house flies in Estonia but virus isolation was not preformed (Herm et al. 2020). Understanding the role that filth flies plays in the transmission of viruses may be critical toward prevention and control of major disease outbreaks.

Surrogate viruses have been used to model virus replication and movement by certain vectors (Mech et al. 2018, Streck and Truyen 2020, Joshi et al. 2016, Leme et al. 2017) when examining the vector competence of insects with pathogens that are not endemic to the area of study. In the present study, Swinepox virus (SwPV) was selected as surrogate for African Swine Fever Virus (ASFV) along with Senecavirus A (SVA), and Bovine Viral Diarrhea Virus (BVDV) as surrogates for Foot and Mouth Disease Virus (FMDV) and Classical Swine Fever Virus (CSFV). These pathogens infect livestock species and are currently not present in the United States but pose a threat to animal production if introduced (Blome et al. 2017, Costard et al. 2013, Pacheco et al. 2015). These surrogate viruses are endemic in the USA and are in the same virus families and/or are similarly structured as the viruses they represent. While Swinepox virus and African Swine Fever Virus are not in the same family they are both double strained DNA virus and were previously classified in the same family because of the increased structure homology. Building off this study and the findings in chapter II, the objectives of this study was to evaluate virus viability in flies exposed to viruses Swinepox, Senecavirus A, and Bovine Viral Diarrhea Virus.

Methods

Viruses and cells

The SwPV NVSL (catalog number 002-PDV) and SVA strain Hawaii were amplified in ST (Swine testis) cells. While the BVDV1 strain singer was amplified in MDBK Madin-Darby bovine kidney (MDBK). Cells were cultured at 37°C with 5% CO2 in MEM medium (Corning) supplemented with 10 to 20% fetal bovine serum (FBS; Seradigm), 2 mM l-glutamine (Corning), 1% Antibiotic-Antimycotic 100X (Gibco), and gentamicin (50 µg/ml; Corning).The virus titration was conducted using the Reed–Muench method (Reed and Muench, 1938). The titers for SVA was about 10^9 tissue culture infectious dose ($TCID_{50}$/ml) while around 10^7 $TCID_{50}$/ml to SwPV, and BVDV.

Flies

The flies used in this study are house flies (*Musca domestica*) in the family Muscidae. They are from a lab colony at Oklahoma State University. The colony was started by combining lab raised flies from Texas A&M and wild caught flies from Stillwater. The flies used for the trials were at least ten generations removed from the initial population. They were housed at 80% humidity and 26.7^0C. The larvae were raised on a mixture of wheat bran, calf mamma, and vermiculite.

Study Design

For the exposure of each virus, 120 newly emerged and unfed house flies were divided into five cages. The size of each cage was 30.5 cm^3, had aluminum screen sides, a mesh stockinette sleeve, and held 24 flies, 12 males and 12 females. Two milliliters of virus solution was placed in a 25 mm petri dish and set inside each cage. The virus solution was removed after

one hour and water and sugar cubes were placed in the cages through the sleeve. Flies from one cage were collected in each of the sampling points: 0, 6, 12, 24, and 48 hours post exposure. To collect the flies, the cage was placed in a -20 °C freezer for at least ten minutes at each time point. The flies were then moved from the cage to microtubes and placed in -80 freezer for later processing.

Virus isolation

Five hundred microliters of PBS (Corning) were added to the flies. Microtubes containing the flies were crushed with a pestle and bead beat twice at 2000 rpm for two minutes. Samples were then centrifuged at 5000 x g for 10min. The supernatant was used as the inoculum. Virus isolations (VI) were conducted in 24 well plates with 70% confluent cells. For each virus the VI procedure was conducted in the appropriate cell line as describe above. A total of 100 µL of the supernatant was incubated and adsorbed for 1 h. The inoculum was then removed from each well and washed 3 times with 500 µL of MEM. After washing 500 µL of complete MEM was added to each well and the plate was incubated for 48 to 96 h. A total of three passages was conducted. Cells were examined daily for cytopathic effect. At the end of the third passage, plates were frozen and thaw and the supernatant was used to extract nucleic acid using the Quick-DNA/RNA Viral kit (Zymo® Research) following the manufacturers protocol. The nucleic acid was then submitted to qPCR (SwPV samples) or RT-qPCR (SVA and BVDV samples).

Nucleic acid amplification - qPCR and RT-qPCR

After the extraction, nucleic acid amplification was conducted using Luna® universal probe qPCR master mix (New England Biolabs) for SwPV and Luna® universal probe one-step RT-qPCR kit for SVA and BVDV. The reactions were prepared following the manufactures recommendations. Two microliters of extracted viral nucleic acid was added to the master mix, containing specific primers and probes as described on Table 7. All samples were ran in an

Applied Biosystems™ 7500 Real-Time PCR (Thermo Fisher Scientific). DNA samples were ran for 1 cycle at 95^{O}C for 60 seconds, followed by 40 cycles at 95^{O}C for 15 seconds, and 60^{O}C for 30 seconds. The RNA samples had an initial hold phase at 55^{O}C for 10 minutes, followed by 95^{O}C for 60 seconds, and then 40 cycles at 95^{O}C for 10 seconds, and 60^{O}C for 30. As a positive control, specific synthetic DNA fragments with 500 base pairs were used (gblocks® gene fragment - Integrated DNA Technologies). Gblocks were diluted in TE in a log10 series. Dilutions were run as described above and enable the estimation of the nucleic acid copy number. Ultra-pure water was used as a negative control. Both the samples and controls were ran on the same plate and in duplicates.

Table 7: Primer and probe sequences utilized for each virus

Virus	Primer Probe	Primers
SVA	5'-/56-FAM/CGGATTAGCGGGTCTCCTCACAAA/36-TAMSp/-3'	5'-GTAGCCAAGAGGTTCAAGATT-3' 5'- CAGTAGACTTCTCGACCTCCT-3'
BVDV	5'-/56-FAM/ACAGGGTAGTCGTCAGTGGTTCGA/36-TAMSp/-3'	5'-TGGCATCTCGAGACCTTTATTC-3' 5'-AGTGGTGAGTTCGTTGGATG-3'
SwPV	5'-/56-FAM/AACATCGAGGACTTTGCTCCGGAC/36-TAMSp/-3'	5'-GTCGTCGGTCGCTGTTAAAT-3' 5'-TGGTTCACCCGGTAGATAGT-3'

Results

Flies were successfully exposed to all tested virus. The SwPV DNA was detected at every time point except in males 24 ours post exposure (Table 8). There was a 75% and 65% decrease in number of viral copies between 0 hours post exposure and 6 hours for males and females, respectively. One sample, females flies 6 h post exposure, was positive in the VI assay. The BVDV RNA was recovered from the females at each time point and males at 0 hours after exposure (Table 8). The only viable virus was detected from male house flies at hour 0 post exposure through virus isolation. The viable virus was confirmed by RT-qPCR after the third passage during virus isolation. The viral RNA was recovered from all the samples for

Senecavirus A (SVA) and the virus isolation showed an increase in viral load for each time point (Table 8). Notably, viable virus was obtained from all SVA tested samples. Cytopathic effect and RT-qPCR demonstrated virus replication.

Table 8: CT to estimated number of virus copies conversion for each virus from flies collected and virus isolation plates if needed for confirmation. * Samples without evidence of virus replication were not resubmitted to qPCR or RT-qPCR.

Virus	Sample	Hour	CT pre VI	Estimated number of copies pre VI	Virus isolation	CT post VI	Estimated number of copies post VI
BVDV	Male	0	30.008	968,989	Pos	20.2865	477,492,250
		6	36.0385	20,701	Neg	*	*
		12	UD	N/A	Neg	*	*
		24	UD	N/A	Neg	*	*
		48	UD	N/A	Neg	*	*
	Female	0	25.9215	13,127,802	Neg	*	*
		6	33.393	111878	Neg	*	*
		12	34.805	45,462	Neg	*	*
		24	35.56	28,088	Neg	*	*
		48	32.7585	167,683	Neg	*	*
SVA	Male	0	22.486	46,633	Pos	13.1645	21,822,536
		6	23.688	21,104	Pos	13.188	21,486,886
		12	26.608	3,075	Pos	13.2185	21,058,941
		24	23.7115	20,779	Pos	13.4615	17,940,207
		48	28.325	990	Pos	14.606	8,432,906
	Female	0	19.474	340,019	Pos	13.31	19,825,571
		6	22.687	40,842	Pos	13.5115	17,358,196
		12	21.785	74,046	Pos	14.1065	11,723,644
		24	22.784	38,311	Pos	14.3085	10,261,203
		48	33.3255	36	Pos	14.2185	10,888,785
SwPV	Male	0	28.3115	13,218	Neg	*	*
		6	30.382	3,081	Neg	*	*
		12	30.8925	2,152	Neg	*	*
		24	UD	N/A	Neg	*	*
		48	33.44	358	Neg	*	*
	Female	0	28.679	10,207	Neg	*	*
		6	30.1835	3,543	Pos	*	*
		12	30.814	2,274	Neg	*	*
		24	33.418	364	Neg	*	*
		48	33.7225	294	Neg	*	*

Discussion

In the events of disease outbreaks mass mortality generally cannot be avoided. Mass mortality management systems all have one issue in common, for a period of time the carcasses are exposed to insects specifically flies. The flies that are attracted to the carcasses are not the common blood feeding vectors that normally in disease transmission cycles (Dubie and Talley 2017). Filth flies like house flies, are in animal facilities and areas where carcasses are found (Watson and Carlton 2005). Previous studies have shown that house flies can carry pathogens from one area to another (Khamesipour et al. 2018; Graczyk et al. 1999; Fasanella et al. 2010). Joshi (2016) detected SVA from house flies on farm that had no history of vesicular disease.

The importance of house flies as vectors for African Swine Fever Virus (ASFV) has been shown (Herm et al. 2020(Oelsen et al. 2018)). Notably, pigs were successfully infected with flies exposed to ASFV (Oelsen et al. 2018). However the time length that viable virus was present in the flies was not evaluated. Since house flies are common in mortality management systems and can carry pathogens, we evaluated the virus viability in exposed flies under an in vitro setting.

Infectious viruses was successfully recovered up to 48h for SVA and less frequently from SwPV and BVDV. Infectious virus was recovered from the BVDV exposed flies immediately following exposure and from SwPV exposed flies 6 hours post exposure. The SVA is known to be a resistant viruses under different situations (Dee et al. 2018) and it also demonstrated long viability in flies. Lysyk and Avtell (1986) collected flies over a three day period and captured flies released 20 km away. If flies flew in a straight line at a constant speed that would be 280m/hour. Combined with the current data, the flies could travel 1.7km transporting infectious SwPV or up to 13.4km if exposed to SVA.

The BVDV, CSFV surrogate, was only viable from male flies immediately after exposure (hour 0). Stewart et al. (1975) showed mechanical transmission of CSFV via *Ades aegypti*.

Additionally, CSFV has been shown to be mechanically transmitted by fomites and biting insects (Blome et al. 2017, Penrith et al. 2011). Our findings suggests that flies can potentially carry viable BVDV virus for a shorter period of time compared to SVA or SwPV, still contributing to move the pathogen into livestock operations.

This study provides evidence that house flies could be a potential vector for any of the viral pathogens tested. It should be noted that only 5 flies from each time point compose each tested sample. Nevertheless viable virus were recovered from at least one sample from all tested viruses. These results clearly indicate that flies exposed to solutions containing infectious virus have the ability to transport viable virus for a variable period of time. In a mass mortality event, hundreds or thousands of flies will be exposed to infected body fluids, probably increasing the chances for virus transport and transmission. Additional studies are required to demonstrate viral transmission from house flies exposed to viral pathogens. A future project could also include other species of flies in the filth fly groups, including *Phormia regina* or *Cochliomyia macellaria* in the family Calliphoridae.

CHAPTER IV

Conclusions

The first study showed that the insects attracted to the animal carcass burial in a shallow trench composting system do not differ from the ones in traditional mortality management system (Rosa et al. 2011, Dubie and Talley 2017). The most common dipteran families are Calliphoridae, Muscidae, and Sarcophagidae and the most common coleopteran families are Silphidae and Staphylinidae. These studies have also shown that certain viruses can serve as surrogates to test the potential of house flies to mechanically transmit highly pathogenic viral strains. Based on these two factors and previous studies we developed a potential risk of fly and beetle families for African Swine Fever Virus (ASFV) (Table 9). The low risk are insects that were captured at the field site but are not known to be associated with carcasses of dead animals. The medium risk are insects that are associated with carrion but are not thought of as common pathogen vectors. This includes insects that could potentially transmit the pathogen mechanically if the virus can survive. The high risk are common vectors of pathogens that feed on blood. These insects have been proven in studies to transmit other pathogens. The final category are proven vectors of ASFV studies demonstrated by Herm et al. (2020) and Olesen et al. (2020) that have established house flies and stable flies as vectors of ASFV. More studies in the area of house flies mechanically transmitting viruses need to be conducted. European Food Safety Authority et al. (2021) lists future projects to improve the understanding of mechanical vector transmission of ASFV. There

are current gaps in knowledge related to filth flies like *Musca domestica* as it relates to the ASFV transmission especially from animal mortality management areas. Future studies should examine filth flies and their role in disseminating viral pathogens away from animal mortality management areas. Our current study is a proof of concept and more work is needed to detail the role house flies play in transmitting viruses. This study also shows the importance of timely management of animal carcasses with the mass emergence of fly larvae. Animal carcasses should be buried or placed in areas that limit the access of insects if timely management is not available. Also, future research should include the role of fly and beetle larvae play in passing certain viral pathogens to subsequent life stages to gain a better understanding of immature stage in vector competence

When combining the virus work and specifically the degree-day model, this demonstrated that even after a certain time with certain temperatures flies could become an issue. Specifically more work is needed to determine if additional management such as applying insecticides to the carcasses as they are placed into the trenches will influence fly development or the decomposition process of the pigs. The time of exposure of animals before placed into an animal mortality management system is very important especially during the active fly season. In scenarios where animals cannot be immediately placed into a compost pile, burial pit or a shallow trench composting area then additional steps will need to be taken to reduce the risk of certain flies from spreading infectious agents into the surrounding environment or other animal production areas.

Table 9: Potential risk of beetle and fly families found during this study for the transmission of African swine fever.

No risk	Low risk	Medium risk	High risk	Proven
	Not known to feed on carcasses	Not yet proven to be common vectors of viruses associated with carcasses	Known vectors of other viral pathogens	Proven to vector ASF
Because we are just starting to understand insect transmission of ASF all arthropods attracted to a euthanasia site are considered at this point.	Dolichopodidae long legged fly Ulidiidae Picture winged fly Chrysomelidae Leaf beetles Cerambycidae Longhorn beetles	Sarcophagidae Flesh fly Anthomyiidae root-maggot fly Calliphoridae Blow fly Culicidae Mosquitos Tabanidae Horse fly Staphylinidae Rove beetles Silphidae Carrion beetles	Muscidae Horn fly	Muscidae Stable fly House fly Argasidae Soft ticks

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