Objective 1: New technologies for management of biting and nuisance flies in organic and conventional systems

a. Novel push-pull strategies (NE, NC, USDA-NE, USDA-FL)
b. Evaluation of improved monitoring systems (USDA-NE, CA, TN, NM)
c. Novel toxicants, biopesticides and delivery systems (TX, USDA-FL, USDA-NE, FL, NE, PA, NM)
d. Non-pesticide management options (mechanical, biological) (FL, NC, NE, USDA-NE, USDA-FL, USDA-TX, PA, TN)

a. Push-pull strategies (PPS)

Push-pull strategies rely on the manipulation of the pest by applying pressures to induce behavioral changes that result in less damage to the crop (Pickett et al. 1997). PPS uses repellents, oviposition deterrents, and antifeedants to push the pest away from the crop. By coupling these agents with other agents such as attractants and traps, reductions in pest populations to below threshold levels may be achieved. Application of PPS is useful in the management of pests in animal agriculture by providing alternative pasture fly management technologies, reducing pesticide use and contributing to a more sustainable production system (Cook et al. 2007).

Among the repellents, DEET, N, N-Diethyl-meta-toluamide, is an efficacious insect repellent for human use. However to effectively repel flies on cattle and horses, DEET required frequent treatments per day, and if concentrations exceeded 50% ai, adverse reactions were observed (Blume et al. 1971). Newly developed synthetic insect repellents undergo an extensive registration process that is not required for many natural products. Several natural products are listed among 31 minimal risk active ingredients exempt from the registration requirements of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (CFR40). The application of these "generally regarded as safe" or GRAS repellents are being explored by producers wishing to reduce or eliminate reliance on pesticides when treating their livestock. Plant derived insect repellents fall into 3 broad chemical categories; alkaloids, phenols and terpenoids (Moore et al. 2007). Terpenoid insect repellents are most common and include a variety of known materials; citronella, limonene, eugenol, neem, and thyme. These compounds are known for repellency against mosquitoes and ticks, and many are also active against flies (Peixoto et al. 2015, Palacios et al. 2009, Pavela 2008, Müller et al. 2009). At issue is that a biting fly repelled from one animal becomes a problem for another unless the fly is removed from the system. As a result, the use of repellents to push pests away, coupled with a lure to attract and remove the pest is the primary goal of PPS.

One approach taken was to apply insecticides to designated trap animals to attract flies from repellent treated cattle. This was successfully demonstrated when stable flies were pushed from cattle treated with geraniol toward cattle treated with insecticide (Boxler et al. 2017). In their study, cattle treated with repellent alone, insecticide alone and the combination of repellent and insecticide carried significantly ($P < 0.05$) fewer flies than the control.

Furthermore, the use of surrogate animals may have utility in PPS. Kinzer et al. (1978) observed marked horn flies were attracted to dark artificial cow shapes, emanating heated water vapor and

CO_2 in the absence of real cattle. In Africa, field observations indicated that stable flies were attracted to the blue and black cloth used for the Nzi trap for the control of tsetse fly (Mihok et al. 1995). In the US blue and black insecticide treated cloth targets have been used to effectively reduce stable fly densities (Foil and Younger 2006). Flies visually attracted to the blue/black color were killed by the insecticide following a 30s exposure. Additional research is needed to determine the feasibility of using similar target designs for other pasture flies, optimizing distances and the number of targets necessary to achieve control. We anticipate that using these technologies in concert will enhance the effectiveness of PPS in livestock systems.

Novel push-pull strategies on Cattle. Incorporating non-insecticidal control options in an attempt to alleviate horn fly burdens on cattle will remain key in combating issues with insecticide resistance. One such approach recently garnering much attention is the use of natural compounds with repellent properties against the fly (Mullens et al. 2009, Zhu et al. 2014). Unfortunately, many available laboratory-based assessments typically provide little information regarding effective dosing rates when products are applied to the animal. As such, residual properties and general effectiveness of promising compounds vary between laboratory and field assessments (Zhu et al. 2014). In fact, it has been suggested that horn fly re-infestation following application of repellent compounds in the field is subject to a number of variables specific to pest population dynamics as well as chemical properties of the products themselves (Mullens et al. 2017).

Controlled on-animal testing. Our goal is to establish a protocol for an on-animal evaluation of repellent compounds in environmentally controlled chambers to determine product longevity and effective dosage rates against horn flies. Artificial infestations of colonized horn flies maintained at the Veterinary Entomology Research Laboratory at New Mexico State University will be used to establish base line data specific to horn fly population dynamics in response to on-animal repellent treatments. Artificial infestation procedures will be experimentally evaluated to assess residual effects against established and migrating horn fly populations, as well as subsequent generational emergences. Baseline data collection and general proof-of-concept will be conducted using geraniol, which has been shown to be effective against horn flies in the field (Mullens et al. 2017). Following initial assessments, procedures will be used to conduct effective dose determination assays for multiple products of interest. Establishing an on-animal laboratory-based procedure to determine effective dosing rates prior to field deployment will help expedite screening procedures for repellent compounds of interest.

Field trials. Preliminary data from Puerto Rico suggests that Essentria IC3, a commercially available blend of rosemary oil, geraniol, and peppermint oil, was highly repellent for horn flies. Such plant-based repellents (e.g. geraniol, catnip oil, palmarosa oil and blends) will be further evaluated in a push-pull system to manage fly populations on pasture cattle in Nebraska and North Carolina.

Previously, the feasibility of PPS for stable fly control was studied using groups of yearling cattle in field conditions typical of West-Central Nebraska. Using the biopesticide, geraniol and the insecticide, permethrin they demonstrated a reduction in stable fly populations. The four treatments were push only (cattle treated with a repellent), push-pull (half repellent and half insecticide), pull only (insecticide alone) and untreated control. Pastures measured 6.8 ha and

cattle were in sight of the other treatments. At this larger scale, it was expected that flies moving from cattle with the repellent will be managed after alighting on insecticide treated cattle. Cattle treated with push-pull, and pull only reduced stable fly numbers ($P < 0.05$) (Boxler, et al. 2017). They concluded that biopesticides offer unique options for stable fly management on pastured cattle.

Under the new project we will continue investigating the overall push-pull management strategy, Nebraska field trials will test repellents and various formulations and application methods to keep stable flies from pasture cattle. Repellents tested will be developed as described in Objective 1c (Biopesticides). Weekly treatments of 500 ml of spray per cow will be applied to the legs and belly of each animal to provide approximately 1 mg active ingredient per cm^2. Evaluations of product efficacy will follow standard protocols.

Similar to NE, field studies will be performed in NC where the horn fly serves as the predominant pest of cattle. The number of horn flies will be counted in the pasture approximately 1-2 hours prior to treatment. Cattle will be treated in a chute for thorough coverage or in a pasture where thorough coverage becomes more problematic. The control group will not be treated. Cattle will be returned to the pasture and the number of horn flies counted approximately 1 hour post treatment, then again once daily on day 1, 2 and 3 post treatment. To be similar to the NE studies, that insecticide treatment will be permethrin or other pyrethroid based insecticide.

Repellant effects on fly dispersal. Prior studies examined horn fly dispersal and how the flies responded to repellents applied to livestock. In North Carolina small groups of young stock on small 2-5 acre pastures were used in a mark and recapture study. Horn flies were marked with fluorescent dyes and released. We learned that horn flies (n = 3000) released from 300 to 1100 meters distance were able to locate cattle in as little as 3 minutes, but often took longer, 21and 38 minutes for the first fly to arrive. At wind speeds of 6 mph, 1.3% of the flies located the cattle by flying against the wind, but when wind speeds were 4.5 mph, significantly more 3.5% of the released flies located cattle by flying against the wind. At this small scale, push pull was expected to be effective because abandoning flies have alternate hosts in close proximity. To study this we treated one group of cattle with 1% geraniol mixed in mineral oil and the horn flies were observed to abandon the cattle or if hit with the spray fall to the ground. After the treatment, marked flies (n = 3000) were released in the pasture with the treated cattle. Marked flies landed on body parts where the spray missed but most flies dispersed from the pasture seeking other cattle. The marked flies arrived at the untreated cattle pasture within 7 minutes. Of the total number of flies released, 8% traveled to the untreated cattle, 227 m distance. It is unknown if the remaining marked flies arrived later or dispersed elsewhere. Mark and recapture studies furthered our understanding of spatial limits for flies to acquire new or alternative hosts.

Future studies will expand PPS to a broader assemblage of pasture pests; the horn fly, face fly and stable fly. Mark and Recapture studies will be conducted to examine dispersal distances of horn flies forced to abandon repellent treated cattle. Topography and vegetative barriers have a likely role in the successful dispersal and acquisition of a new host by flies (Fried et al. 2005). Horn flies will be collected from cattle, marked with fluorescent powders and release in the vicinity of repellent treated cattle. Untreated herds located at distances of 1 to 3 miles will serve

as recipient animals. Placement of recipient herds with careful attention to topography and barriers will provide information on the probability of flies acquiring hosts under such conditions. Collected horn flies from the recipient animals will be examined for color markers to establish maximum repellency distances.

Although many essential oils and fatty acids are primarily considered natural repellents, some of these natural repellents cause mortalities in treated insect populations. These dual action compounds will be further addressed in subobjective 1c.

b. Evaluation of improved monitoring systems

Pest fly populations must be quantified in some manner so we may evaluate the effects of experimental treatments. Furthermore, pest monitoring a key to the successful IPM program because observers must know when economic thresholds have been exceeded. As a result monitoring flies usually relies on visual observation of either the insects themselves or quantifiable indicators that insects are present. To avoid subjectivity, or observer variance, quantitative monitoring are considered more reliable. House flies within barns can be effectively quantified by counting the fly fecal and vomit drops left on index cards placed inside livestock facilities (Lysyk and Axtell 1986). Hogsette et al. (1993) effectively quantified flies using sticky cards. While these techniques effectively measure house fly populations inside a barn, better methods to measure fly populations out of doors are needed. Using baited outdoor traps provide a valuable estimate of fly populations but require time to work (Geden 2005). The Scudder grill has served as an easily deployed device to sample house fly populations (Scudder 1947). In a study of flies associated with UK landfills, Lole 2005, compared sticky traps to the Scudder grill. While the Scudder grill was easy to use as an immediate assessment of the fly population, it did not allow for species determination. Furthermore other variables caused dramatic fluctuations in fly counts precluding its usefulness as a long term monitoring tool. In contrast, using sticky cards required more time to process and identify the specimens.

More subjective counting methods are those that count insects on animal predilections sites; stable flies on the legs of cattle and horn flies on each side of animals. While researchers routinely use these monitoring methods, use by producers is frequently unreliable. User friendly methods for monitoring pest populations are needed to allow farmers to implement control measures at the proper time and provide them with a durable record. Recently digital photography is recognized as a tool that may facilitate fly counts on animals and allow the observer to have a more durable record of the fly densities referenced to date.

Development of visual recognition software (Flyspotter®software) to automate the counting of speck cards has significantly reduced the time required to establish population thresholds (Gerry et al. 2011). Development of additional recognition software technologies to quantify flies would be a great benefit to the industry. Currently horn flies are most often counted visually by a trained observer. Studies comparing counts of trained observers and photographs indicate that trained observers can consistently provide reproducible estimates of horn fly densities, and do so much less expensively (Castro et al. 2005). However, this work was done with relatively low fly populations (<200). Studies conducted in NC in the summer of 2010 indicated that the time required for a trained observer to estimate fly densities on animals was about 1 minute per

animal (fly numbers >500). Digitally photographing animals required about 3 minutes per animal, and counting flies on the digital images required another 30 minutes per animal, for a total of about 300-fold more time than visual field observations alone. Recently, Mullens et al. (2016) noted that counting photographed flies took 10-20 times longer than visual estimates and visual counts overestimated fly densities. This is a clear example of the need for improved technology to facilitate quantification of horn fly populations when numbers are high.

Estimating horn fly numbers on cattle with digital photography may be feasible using recognition software provided there is sufficient contrast between the flies and their host. Imaging software ImageJ® https://imagej.nih.gov/ij/ has been used to count ticks and horn flies on cattle in Brazil with a 90% accuracy (Cortivo et al. 2016). The following link references a technique that was used to count dots on any photographed surface and using image processing software (http://reuter.mit.edu/software/dotcount). The limitations would be having to eliminate any part of the picture that is not part of the host or flies prior to counting (because the whole image of an animal will include background images as well) and if the animal is dark or shaded, the flies may not contrast well enough for the software to distinguish them from the host. The MIT software provides the observer a direct comparison of the actual photograph to the negative image and allows for enhancing the contrast to illustrate the desired object to count (Figure 1). The image software can be manipulated to improve the accuracy and eliminate noise. Image processing software will be explored as a tool for counting flies on animals.

See attachment for Figure 1

Figure 1. MIT software DotCount (http://reuter.mit.edu/software/dotcount/) was used to estimate the number of horn flies on one side of a dairy cow. The human estimate was 1100 flies, the DotCount estimate was 791 in black areas (shown on right) and 181 in white areas (not shown) for a total of 972 flies, and the projected grid count was1053 horn flies.

Laboratory evaluation of sensor position and sampling area for insect monitoring system. Partially automated insect surveillance equipment has been around for many years; however it has traditionally relied on the analyzation of video recordings or the use of microphones, making sex and species discriminations nearly impossible. These systems almost always relied on some human interaction to guide the identification process. In contrast, recent work has demonstrated that wingbeat sensors can accurately detect and categorize insect species (Batista et al. 2011, Hao et al. 2013, Chen et al. 2014, Silva et al. 2015), with sensors successfully distinguishing insects of up to five different insect classes with greater than 96% accuracy and with up to ten insect classes with greater than 79% accuracy (Chen et al. 2014). Wingbeat sensors sample a specified air space by emitting infrared light from one side of the sensor apparatus. This light is then detected by a complimentary row of detectors. When an insect passes across this plane, the emitted light is blocked by the wings and body of the insect in flight, resulting in fluctuations in light intensity which is analogous to the wing beat frequency of the insect in flight. This output is then passed through a machine learning algorithm which (based on previously built models) can classify the detected insect into specific classes (species).

While much of the computational difficulties surrounding the use of an automated system have been dealt with, there is still much work to be done concerning the design and placement of the

physical sensor within an insect monitoring system, particularly if multiple insect species are to be simultaneously identified. Thus, sensors must be designed to detect the largest diversity of pest species. In order to create a multi-species efficient physical senor system, some basic design and placement criteria must first be determined. To address this, we propose to evaluate in the laboratory several monitoring system designs to identify 1) the optimal sensor orientation for detection of multiple pest species of interest, and 2) the maximum sampling area that over which sensors will correctly function. We will then use the best monitoring system design to determine optimal placement of monitoring devices at commercial animal facilities.

Field evaluation of monitoring system placement at commercial animal facilities. Robotics and unmanned aerial vehicles (drones) may become important tools in livestock pest management. The efficacy of drones for monitoring cattle and wildlife was studied by the USDA ARS Cattle Fever Tick Research Lab in TX. They determined that drones could be a useful tool in the monitoring program by providing access to rugged terrain and increase inspector safety (Goolsby et al. 2016). In their study, the drones were flown at an altitude of 50m. Flying drones closer to the ground could be a viable sampling strategy for pasture flies or for flies around farm buildings. Hogsette et al. 1993 collected more house flies on sticky cards placed 0.5 m above the floor than a higher location 1.8 m above the floor inside a poultry house. Similarly, in outdoor settings, house fly catches decreased above 1.5 m, suggesting that any drone sampling should be done relatively close to the ground (Zahn and Gerry unpublished data). Drone sampling of livestock pests will be explored to determine feasibility and to determine optimal flight plans for optimization.

c. Novel toxicants and delivery systems

Resistance to currently available insecticides remains a major problem for the control of house fly and horn fly in the United States. Resistance to pyrethroid insecticides has been detected in stable fly populations in Florida in recent years as well (Pitzer et al. 2010; Olafson et al. 2011). New insecticides with novel modes of action can be very useful in managing resistance problems. Novel toxins and delivery systems will be tested for their utility in fly control. Historically, pest control for Public health and animal health benefited from insecticides developed by major chemical companies for control insect pests of crops. There are several novel insecticides that are in the process of EPA registration for crop pests, including a number of new molecules (such as chlorantraniliprole and cyantraniliprole) of the diamide chemical class. These molecules are ryanodine receptor agonist, causing impairment of insect muscle function which results in rapid cessation of feeding (Annan et al. 2011). A new insecticide (SYP-9080, chlorantraniliprole) with similar mode of action has also been developed. IPP-10 and cycloxaprid are two new neonicotinoid insecticides. Another novel insecticide, isoxazoline, is a potent blocker of insect ligand-gated chloride channels (Ozoe et al. 2010). Samples of these novel insecticides will be obtained and tested on the three major fly species (the house fly, horn fly and stable fly) using established bioassay techniques. A recently published study found no cross resistance between these novel molecules and currently used insecticides in the whitefly (Li et al. 2012). This is encouraging in term of resistance management. Other novel insecticides that are currently being registered or have been registered in recent years for controlling crop pests, such as pyrifluquinazon, sulfoxaflor, novaluron, methoxyfenozide, clothianidin, and flonicamid, will also be evaluated for fly control when possible. Commercial insecticide

products with new modes of action, formulations, or delivery methods will be field tested against a range of fly species using standard techniques and delivered through objective 5 stakeholder and community engagement.

Biopesticides have received more attention in recent years (Geden 2012). Essential oils have been evaluated as insecticides for the control of various insect pests (Regnault-Roger et al. 2012). There are an increasing number of studies demonstrating the efficacy of essential oils and oil blends (Essentria) for fly control (Lachance and Grange 2014, Mullens et al. 2017). Zhu et al (2010) reported repellency and toxicity of the catnip oil against the stable fly. Essential oils are effective against the myiasis-producing fly, *Lucilia sericata*, in Egypt (Khater et al. 2011). Lab trials using various olfactometer designs will test potential biopesticides as fly attractants and repellents and the duration or longevity of each compounds activity period. Repellents also testing positive as contract toxicants will as needed undergo mode of action studies in collaboration with the UNL toxicology lab. Before advancing to field trials (objective 1a), biopesticides will, if needed, be formulated in collaboration with ARS scientists at Peoria, Illinois.

Delivery systems. We will continue research in novel delivery of pest control products by acquiring and testing new materials from collaborators within USDA-ARS, universities and international collaborators. Enhanced control also can be achieved through development of new insecticide formulations and/or delivery systems. We will continue to work with industry partners to develop more efficient and safe insecticide delivery systems for cattle ranchers in the U.S.

In field trials stable flies were highly attractive to CO_2, and white targets (Zhu et al. unpublished data). It is clear that flies use both visual and chemical cues to identify hosts and resting sites. Attractants developed within the Biopesticide section of this objective will be incorporated into traps for use in stable fly push-pull management strategies. Traps will be designed to incorporate both visual and olfactory stimuli and target host and resting site seeking behaviors. Field testing will be done in Nebraska and Florida.

Autodissemination of pyriproxyfen. Pyriproxyfen (PPF) is a juvenile hormone analog that inhibits pupal-to-adult metamorphosis when applied to larval habitats (Invest & Lucas 2008, Seng et al. 2008). It has high activity against immature dipterans including mosquitoes and some flies (Hatakoshi et al. 1987, Kawada et al. 1987, Bull & Meola 1994). Although PPF can be applied as a broadcast larvicide, such treatments are labor-intensive and can have unintended effects on non-target species. Recently it has been shown that pyriproxyfen can be disseminated to aquatic habitats of mosquitoes by the adult females themselves; both in the laboratory (Gaugler et al. 2011) and field (Devine et al. 2009). In this "autodissemination" approach, adult female mosquitoes pick up a dust payload of PPF at stations, and transport that payload to egg-laying sites where the PPF is deposited along with eggs. The result is pinpoint delivery of a larval control product at the point where is it needed, and the results have been impressive (Devine et al. 2009). House flies have been found to be highly sensitive to PPF as well, and early testing demonstrated that adult house flies can be used as autodissemination vehicles to transport PPF to fly larval breeding sites (Geden & Devine 2012). Higher-potency formulations have been developed and the compatibility of PPF with pupal parasitoids has been established (Biale et al.

2017). For this project, the performance of PPF in different fly breeding substrates will be evaluated and autodissemination stations will be developed that will lure flies into a device where they self-treat with PPF and leave the station to contaminate larval development sites.

House flies, stable flies and face flies are commonly seen resting on various surfaces following feeding. These resting behaviors provide an unconventional control opportunity using insecticide treated targets and resting sites. Such control strategies may be designed to prevent insecticide exposures for humans and animals. The USDA CMAVE laboratory in Gainesville, FL will evaluate the efficacy of Vestergaard-Frandsen (VF) insecticide-treated fence for management of stable flies and other nuisance flies. In this attract and kill study, animals and structures to be protected from flies will be surrounded by the VF treated fence. Efficacy will be estimated by having comparable untreated control situations with monitoring devices (e.g. traps) inside and outside of the enclosures. Potential study sites include the National Zoo in Washington, DC, a dairy farm near Lincoln, Nebraska, and an Exotic animal rescue unit near Gainesville.

Evaluation of toxic and non-toxic sugar baits for management of stable flies. For these studies candidate sugar baits will be evaluated in the laboratory and under semi-field conditions. Promising bait combinations will be evaluated in the field. Evaluations will be based on increased attraction by the bait when compared with similar situations without the bait. Potential study sites: Initial site will be in Gainesville, FL, with other candidate site selected during the course of the project.

d. Non-pesticide management options

MECHANICAL

Traps have been used for years to monitor, and in some respects reduce, stable fly populations. Such traps serve as an optical attraction for stable flies with varying efficacy (William 1973, Broce 1988, Beresford and Sutcliffe 2006). Preliminary studies begun in 2017 will continue into 2018-19 to determine if the Knight Stick sticky fly trap can be transformed into an attract and kill device by the addition of D-Terrance fabric from Vestergaard. Preliminary tests will be conducted to determine the effects of open structures around the trap. This will be followed by the addition of fabric in various configurations to determine how to maximize trap attraction and limit interference by the fabric.

Bruce (1940) was the first to publish plans for a passive horn fly trap that consisted of a screen covered wooden frame sufficient in size to allow cattle to pass through. Curtains suspended from one end brushed flies from the animals as they passed through. Fleeing flies were captured in the screened hollow walls (Bruce 1940, Hall and Doisy 1989). Tozer and Sutherst (1996) modified the trap design with a translucent top to increase efficacy by increasing ambient light within the trap. This Australian Fly trap was more efficient than the Bruce trap. Similar fly traps continue to be used by producers with mixed results stemming from altered fly behaviors. Moreland et al. (1995) patented a modified Bruce fly-trap by adding a rigid canopy and black lighted electrified grids on the ceiling and side-walls. A centrally suspended curtain brushed flies from the surface of the animal as it passed through. For a time, disturbed horn flies, attracted toward the black lights, were killed in the electrocution grids (Watson et al. 2002).

Although the trap significantly reduced horn fly densities, the cost (>$10,000) was unacceptable to producers (Surgeoner et al. 1998, Watson et al. 2002).

Researchers at NCSU developed a unique vacuum pressure walkthrough fly-trap that physically removes flies from the cattle and the air surrounding the cattle as they pass through (Denning et al. 2014). Using this device, horn fly densities were kept below threshold levels for 14 weeks during peak horn fly season without the use of insecticides. Studies in North Carolina have demonstrated horn fly control with traps and these cattle have been insecticide free for 10 years.

Further study is needed to explore the efficacy of this trap for other species, particularly the face fly and stable fly. Regional efficacy studies demonstrating pasture fly control for milking herds for all three pasture flies are needed. Economic analyses are needed was well as comparative studies with similar devices.

Comparative study of the CowVac and Bruce walk through fly traps for the control of pasture flies was completed in Minnesota (Kienitz 2016). Their goal was to determine the efficacy of each system for horn fly, face fly and stable fly, and weigh the benefits against the cost of each trap and its upkeep. CowVac traps was purchased for research from Spalding Labs, Reno, NV. Construction of the Bruce fly trap was performed following design schematics and assembled on site. The Cow-Vac reduced horn flies on cows by 44% with no significant differences in milk production or somatic cell counts. Additional studies are needed where fly populations are greater and fly seasons are longer.

CowVac fly traps are currently located on 5 certified organic dairies in NC. Additionally, CowVac fly traps are also located on three dairies without organic certification in NC and three more in Florida. The southern horn fly season commonly falls between March and November, with horn fly densities above 200 flies per animal for a significant period. We propose to conduct a similar study to that of Kienitz (2016) in the southern region to determine the efficacy of the CowVac fly trap on horn fly, stable fly and face flies on organic and non-organic dairy farms. The traps will be used twice daily when cattle are brought in for milking. Flies will be counted on the cattle once each week by a trained observer. In addition, cattle will be photographed with high resolution digital cameras and the images downloaded for counting using the MIT software DotCount (http://reuter.mit.edu/software/dotcount/) describe previously. Furthermore all dairies participating in the study are in the DHIA Program. Through the Dairy One Cooperative (http://dairyone.com/dhia-record-services/about-dhia/) DHIA record services we will serve as the data source for milk, fat and protein production and SCC (somatic cell counts) as recorded from monthly DHIA testing. Body weight and BCS (body condition scores) were recorded bi-weekly as cows exited the milking parlor.

Sticky Trapping. Lab and field trials will be done to improve capture rates of stable flies on sticky traps. A series of trials using white panel or knight stick traps with olfactory attractants added to increase trap capture rates will be done. Promising olfactory attractants and formulations identified in laboratory assays will be tested against native Nebraska flies in the field. Trials will compare replicated pairs of traps with and without olfactory attractants in pastures with cattle. Capture rates will be monitored daily for five consecutive days.

Traps have been used for years to monitor and in some respects reduce stable fly populations. Such traps serve as an optical attraction for stable flies with varying efficacy (Williams 1973, Broce 1988, Beresford and Sutcliffe 2006). Preliminary studies begun in 2017 will continue into 2018-19 to determine if the Knight Stick sticky fly trap can be transformed into an attract-and-kill device by the addition of D-Terrance fabric from Vestergaard. Preliminary tests will be conducted to determine the effects of open structures around the trap. This will be followed by the addition of fabric in various configurations to determine how to maximize trap attraction and limit interference by the fabric.

BIOLOGICAL

Integrated pest management includes biological control and resistant varieties as key components. For biological control a variety of agents; parasitioids, predators, bacteria and fungi top the list. Several strains of the entomopathogenic fungi, *Beauveria bassiana,* have been tested for control of filth flies in agricultural systems. Several *B. bassiana* products that are commercially available and labeled for house fly control, produced mixed results in the field. Under this multistate project, microbe and insect interactions are in part focused on bacteria that cause enteric disease in humans, bacterial infections of the mammary or eyes of cattle or how insect avoid infections from bacteria (obj 3). Biological control of filth flies has traditionally used beneficial insects and bacteria as tools to manage pests. Under this subobjective we will examine how pathogens may be used to control pests, interact with non-target insects, how to optimize their benefits as biological tools, and develop resistant varieties.

Entomopathogens. Several strains of the entomopathogenic fungi, *Beauveria bassiana,* have been tested for control of filth flies in agricultural systems. However, balEnce™, the *B. bassiana* product that is commercially available and labeled for house fly control, has had mixed results in the field. Laboratory studies have demonstrated that although the balEnce strain of *B. bassiana* (HF23) is highly pathogenic against house flies, the formulated product contained few viable conidia and the product failed to perform better than a control treatment. The same lab-based studies identified another strain (GHA) as highly pathogenic against house flies (Weeks et al. 2017). The strain is available commercially in two formulations: Botaniguard ES and the Organic Materials Review Institute (OMRI) approved Mycotrol O. Another product, MET 52 EC, containing the *Metarhizium anisopliae* strain F52 was also found to be pathogenic (although not OMRI approved). In application assays it was found that these two formulations were effective at increasing mortality in houseflies when applied as a bait (Machtinger et al. 2016) and when applied to an oviposition substrate they reduced survival of larvae (Machtinger et al 2016). However, it was noted that less eggs were laid on the fungi-treated surfaces.

Laboratory experiments will focus on attempting to understand the oviposition and feeding deterrence that appears to occur and determine if the flies can sense the fungi or if the repulsion is related to the formulation. In order to do this, blank formulations will be obtained and tested against the formulated fungal products. Additional laboratory experiments will test these products with horn flies and face flies. Further experiments will be conducted on application methods. As both horn flies and face flies are most likely to be controlled through contact with treated animals, these strains will be tested by exposing flies to treated cattle hide or cattle hide substitute. The duration of activity could be determined by exposing the cattle hide to sunlight and evaluating the effect on fungal pathogenicity with increased UV absorption.

Following on from laboratory trials, fungal products that achieved successful control of flies in bioassays will be evaluated in livestock operations with nuisance fly problems. The effectiveness of the fungi at reducing fly numbers will be evaluated through the use of a suitable monitoring method for each fly species, before, during and after the treatment. On cattle farms the efficacy of baits will be tested by monitoring the effect on the resident house fly population with Scudder fly grids and sticky ribbons, before, during and after the treatment. Where situations allow baited traps will also be deployed. Field trials on equine facilities will test the application of the treated dust to bedding for stable flies. On equine farms, the emergence (%) of adult stable flies will be monitored with emergence traps from both treated and untreated bedding and feeding sites known to produce stable flies. The effect of the treatment on the population will be monitored by counting the number of stable flies landing on the lower legs of horses in both treated and untreated stalls. Samples of larvae, pupae collected from breeding sites and any adults that eclose from collected pupae will be monitored for mortality and sporulation. The application of fungal formulation as liquids or dusts to cattle will be evaluated for horn flies and face flies. Any field work on face flies will be completed in collaboration with a state where they are an economically important pest. On-animal sampling of both horn flies and face flies are conducted by counting the number of flies on animals. Samples of flies will also be regularly taken following treatment to monitor the number of flies infected with fungi. Flies will be taken to the laboratory where they will be allowed to die naturally and then observed for sporulation.

Finally, a *B. bassiana* strain (EN1) was collected from a Florida horn fly and is currently maintained in the laboratory. In laboratory evaluations, this strain has been found pathogenic to horn flies. The strain represents the first U.S.-collected *B. bassiana* strain from horn flies and the second reported in the literature. We will select for increased virulence and evaluate the efficacy of the enhanced EN1 strain against these flies using similar protocols.

Improved fly control on poultry facilities with microbial products. The poultry industry is an important part of U.S. agriculture. Many of these facilities experience house fly pressure that can lead to losses in production and increase food safety concerns. However, the rapid development of insecticide resistance by flies, even those with novel modes of action such as spinosad and imidacloprid, has led to an imminent collapse in producers' ability to manage house fly pests. Throughout the country, flies are now resistant to the QuickBayt and QuickStrike products that were producers' last line of defense for fly control for many years. New fly control tools are desperately needed, and they must be environmentally safe as well as effective and economical.

Microbial control of adult flies using fungal pathogens is a highly attractive alternative for managing insecticide-resistant flies. The pathogenic fungus *Beauveria bassiana* is particularly promising because spores have a long shelf life and can be formulated as a bait or applied using conventional insecticide application equipment as a liquid spray. The spores germinate on the outside of the fly, penetrate the fly cuticle, and kill the fly several days later. It has no harmful effects on birds, no environmental issues, and is thought to be compatible with natural enemies of the flies. To date only a single *B. bassiana* product has been labeled for fly control, under the trade name balEnce™. Sadly, this product is not as efficacious as desired and the long time that it takes to kill the fly (6 days) limits its effectiveness during fly population surges.

A better *B. bassiana* tool can be developed for the poultry industry, which would be an important addition for integrated pest management programs, as well as be compatible with organic farming practices. The objectives of this project are to 1) collecting new fungal isolates from flies on Pennsylvania and Georgia poultry facilities and screening the isolates to identify strains with faster time-to-kill properties; 2) testing the most promising strains, and subjecting them to selection to further improve time-to-kill; 3) ensuring their compatibility with the most important natural enemies of house flies (two species of parasitic wasps, and the beetle predator *Carcinops pumilio*); and 4) developing novel autodissemination devices for fly control.

Another project will concentrate on *B. bassiana* use in poultry facilities in Florida and Pennsylvania. In the first phase, surveys will be conducted to obtain a variety of new isolates from flies collected from poultry facilities. These isolates will be screened for efficacy, with particular emphasis given to strains with fast kill rates. In the second phase, several promising isolates will be chosen and selected for 10 generations to reduce the time to host death. LT_{90}'s of selected and unselected isolates will be compared after the 10^{th} generation. In the final phase, the most promising isolate will be tested for compatibility with fly parasitoids, and novel methods will be devised for delivering conidia to flies in the field.

Parasitoid-Beauveria bassiana-house fly interactions. Under this sub-objective we will compare the LC_{90}'s of *B. bassiana* in flies and some of their principal parasitoids when both are applied directly to the insects. Use of this pathogen in the field will inevitably result in circumstances where fly larvae acquire the infection and then pupate. Such infected pupae appear healthy and normal to the human eye but rarely produce an adult fly. The effect of such pupae in the environment on pupal parasitoids is unknown. We propose to first determine the fate of parasitoids (*Muscidifurax raptor, M. zaraptor, Spalangia cameroni,* and *S. endius*) that are placed in *B. bassiana*-infected fly immatures when adult female wasps are only given infected hosts for oviposition. We also will assess whether female parasitoids become infected when they feed on or oviposit in infected host pupae. We will then evaluate whether female wasps can detect and refrain from ovipositing in infected hosts. Such discrimination would improve the compatibility of *B. bassiana* with parasitoids.

Evaluation of Pseudomonas protegens. During the previous project, several bacteria species (*Pseudomonas protegens, Phtorhabdus temperata, and Serratia marcescens*) were tested in combination with *B. bassiana* to determine whether the cuticular insult caused by fungal penetration would allow entry of faster-killing bacterial pathogens. Although the desired synergy was not observed in any of the combinations, *P. protegens* was observed to have surprisingly high virulence when applied topically (Johnson 2017). During the course of conducting these assays the culture medium in which *P. protegens* was grown had an immediate toxic effect when applied topically. Little is known about this species, but it is regarded having plant-protecting properties and an associated insect toxin known as FitD (Péchy-Tarr et al. 2008). This toxin is known to be active against *Drosophila* but has not been evaluated against muscoid flies (Rangel et al. 2016). We propose to conduct evaluations of *P. protegens* and its associated toxins against adult and larval house flies and stable flies. If larval efficacy is high we also determine non-target effects on parasitoids of the genera *Muscidifurax* and *Spalangia*.

Heat tolerance in different geographic strains of parasitoids. In the previous project, colonies of *Muscidifurax raptor, M. zaraptor, Spalangia cameroni,* and *S. endius* were established from collections made in Minnesota, Nebraska, inland southern California, and Florida. Initial screening of these isolates for heat tolerance revealed little evidence for difference among the collections. This bioassay had issues, however, in that the time course of observation was limited to 24 hours and the "hot" temperature regime may have been too hot, as performance of all strains under hot conditions was poor. We propose to revisit this question by choosing a more moderate high-temperature regime for the bioassays and by holding the parasitoids for an entire generation (rather than 24 hours) for the assessment.

Pest resistant cattle. Cattle producers breed animals for specific traits (e.g., weight gain), and some companies are marketing animals as horn fly resistant (HFR) which is more accurately described as a low-fly carrying cow (LFC). More specifically, *Bos indicus* cattle and European breeds of *Bos taurus* (e.g., Charolais and Chianina) are considered HFR and consistently carry lower numbers of horn flies compared to British cattle breeds (Steelman et al. 1991; Steelman et al. 1993); this natural resistance is a heritable phenotype (Brown et al. 1992). In collaboration with other regional hatch project members, USDA-MARC, and stakeholders we are working to improve horn fly phenotyping procedures and to identify the genomic regions responsible for HFR. Cattle in Nebraska, Arkansas, and North Carolina will be phenotyped and genotyped as resistant (low carrier) or susceptible (high carrier). Novel phenotyping procedures will be developed that include traditional entomology, animal science, agriculture economics, and digital image processing with computer learning. The outliers (high/low carriers) identified in each herd will be genotyped using Neogen's Bovine 250K SNP chip, and genomic regions associated with HFR will be identified using genome wide association studies. Expected outcomes include the identification of genetic markers, improved phenotyping procedures, and a value for HFR animals. We will then synthesize this information into a stakeholder-friendly mobile application that permits easy identification of phenotypes thereby validating the purchased/selected genotype.

Objective 2: Insecticide resistance detection and management
a. Assessment of insecticide resistance (CA, FL, NE, NY, TX, USDA)
b. Leveraging the Stomoxys and Musca genomes for novel control measures (NY, USDA)

a. Assessment of insecticide resistance

Accomplishments during current Multistate Project.
Over the last five years several groups have documented issues associated with insecticide resistance in house fly (Gerry and Zhang 2009; Li et al. 2013; Rinkevich et al. 2013; Scott et al. 2013; Højland et al. 2014; Kavi et al. 2014; Seraydar and Kaufman 2015; Sun et al. 2016; Kasai et al. 2017; Scott 2017; Sun et al. 2017). Especially notable were investigations into the mechanisms of resistance to neonicotinoids (Kavi et al. 2014) (including the potential role of behavior (Gerry and Zhang 2009; Seraydar and Kaufman 2015)), identification of new Voltage sensitive sodium channel (Vssc) mutations causing high levels of pyrethroid resistance (Sun et al. 2016; Kasai et al. 2017; Sun et al. 2017), investigations into the frequency of Vssc mutations in field populations (Rinkevich et al. 2013) and fitness cost associated with the resistance

mutations (Rinkevich et al. 2013). Also noteworthy is the completion of the house fly (Scott et al. 2014) and stable fly genomes by members of this multistate project (and others). Specific examples of previous work are given below.

Selection of field collected house flies with imidacloprid resulted in a strain with >1000-fold resistance. The resistance has a significant fitness cost under laboratory conditions (Kavi et al. 2014). Resistance was due to two factors, one on chromosome 3 and another on chromosome 4 (Kavi et al. 2014). Studies aimed at identifying the genes responsible for this resistance are underway.

House flies evolve resistance to pyrethroid insecticides due to mutations in Vssc. Three Vssc alleles are known to confer resistance to pyrethroid insecticides: kdr, kdr-his and super-kdr. Work from this multistate project has recently identified two new mutations (D600N and T929I) which confer high levels of resistance to pyrethroids (Sun et al. 2016; Sun et al. 2017). This is an important discovery, which opens the way to investigations of the frequency of these new resistance alleles in populations across the USA.

A nationwide survey for permethrin resistance in stable fly populations was conducted from 2013 to 2015. In this survey, we provided diagnostic dose kits to researchers located in nine states. Of these participants, ultimately, 13 farms in five states were successfully sampled for stable fly resistance to permethrin. Our results indicate that these populations are considerably resistant and that producers may be experiencing a lack of stable fly control following animal treatment with permethrin. Resistance was lowest in western Nebraska and eastern Minnesota. The stable fly Vssc kdr-his allele is associated with increased levels of pyrethroid resistance in a laboratory-challenged strain, and the allele was detected at high frequency in field populations from Tennessee, Florida, and Washington (2012, 2014). As part of the 2015 nationwide survey for resistance to permethrin, stable fly specimens from Louisiana, Minnesota, and Nebraska that survived permethrin challenge at 3X and 10X-LC99 were screened for frequency of the kdr-his allele. For each location, there did not appear to be an observable increase in frequency of the kdr-his allele associated with the increasing challenge dose. Other, unidentified Vssc alleles and/or alternative mechanisms may account for these results. The number of survivors from LA, MN, and NE was low at 3X ($n < 20$) and lower still at 10X ($n < 7$). Stable flies from two locations in Modesto, California were challenged at 10X with similar low numbers of survivors, yet all ($n=10$ and $n=6$) were homozygous for the kdr-his allele. The CA results possibly reflect stable fly populations under more intense insecticide pressure.

Planned research for next multistate project.

House flies. Resistance monitoring efforts will again be carried out in many states with an effort to, establish baseline susceptibility to new fly control products, document resistance levels, reversion of resistance and the evolution of resistance to current insecticides, as well as new insecticides that become available for fly control over the next five years. In addition to using bioassay methods, molecular techniques (sequencing of PCR products, multiplex PCR, etc.) will be used to evaluate the frequency of important resistance alleles in house fly (Rinkevich et al. 2007; Kozaki et al. 2009). This two-pronged approach helps to not only document the level of resistance found, but also the underlying causes. In addition, studies will be carried out to

determine mechanisms of resistance to neonicotinoid insecticides. Specific examples are given below.

Previous work from this multistate project found two new Vssc mutations (Kasai et al. 2017) and these were confirmed to confer high levels of pyrethroid resistance (Sun et al. 2017). Thus, it is critically important to monitor for these mutations in field populations such that resistance management strategies can be implemented before control failures are observed. We will collect house flies from animal production facilities across the USA and examine the levels of pyrethroid resistance as well as the frequency of the Vssc mutations found. Facilities having high levels of resistance will be provided with alternative control strategies.

The mechanisms of imidacloprid resistance in the KS8S3 strain will be investigated using a comparative transcriptomic approach. Candidate genes identified by this approach will be validated by expressing them in transgenic Drosophila melanogaster under the control of a UAS promoter. Once the mutations responsible for the resistance are identified and validated we will examine the relative frequency of these mutations in field collected populations that will also be tested to determine their level of imidacloprid resistance.

Next generation sequencing has dramatically increased our capacity to identify genetic variants associated with specific phenotypes, including pesticide resistance (Rinker et al. 2016). We have sequenced the genomes of two pyrethroid resistant strains, LPR and A3, and identified genetic variants that differentiate it from the susceptible genome reference strain, aabys (Scott et al. 2014). We confirmed the existence of the kdr allele in both strains, and we will use the data to identify other candidate alleles that could be associated with resistance. Additional genome sequencing of susceptible and resistant strains and pools of resistant and susceptible flies will also be performed. We will test promising candidate alleles with functional experiments that use targeted allele replacement (Heinze et al. 2017) or transgenics in D. melanogaster.

Work will continue on the behavioral resistance to imidacloprid. We have selected resistant lines in an effort to confirm the existence of behavioral resistance. The next step will be to conduct additional behavioral assays and neurophysiological assays that will point us toward the receptor(s) that are driving this behavioral resistance. Finally, a genomics approach will be utilized to identify the altered alleles that support this phenotype. Ultimately we hope to identify the mutation responsible for this mutation and determine its frequency in natural populations.

As novel insecticides are introduced for fly control (see Section 1c for examples), it is necessary to generate baseline susceptibility on fly populations and then continue to monitor so as to rapidly identify when resistance begins to evolve. We will do this for abamectin and for additional compounds as they come to market.

*Stable flies.*We will continue to collect data on stable fly permethrin resistance through the distribution of resistance kits. Our goals include 1) expansion of sampling to states where sampling has been lacking and 2) to follow multiple farms within a season where permethrin is used to assess changes within a season.

The original University of Florida permethrin-susceptible stable fly colony (UFD) was established in 2007, thus we currently are establishing a new stable fly colony (Pitzer et al. 2010). We will expose a subset of this newly established colony to permethrin selection pressure to generate a 2nd permethrin-resistant strain. Comparisons will be made between these two newly established strains for the presence of the kdr mutation, and, should we move resistance to a higher level than the original permethrin resistant stable fly strain we will identify if additional resistance mechanisms emerge, such as metabolic resistance or additional sodium channel mutations.

Permethrin is a "Type I" pyrethroid widely used on cattle to manage fly and tick pests. Other types of pyrethroids exist, but little information has been published on their efficacy against stable flies. Therefore, we will establish toxicity and cross-resistance profiles for at least one Type II pyrethroid, such as cyhalothrin or cyfluthrin.

Stable fly specimens collected from multistate project members over the past several years as part of field collections and the nationwide survey for permethrin resistance will be used to screen for the presence of other Vssc mutations that may confer permethrin resistance. This will be accomplished using amplicon sequencing (AmpSeq), a PCR-based approach that allows multiplexed sequencing at hundreds of genomic locations simultaneously. Because the output is nucleotide sequence from DNA templates, AmpSeq provides a means to identify both known single nucleotide polymorphisms (SNPs) and new genetic variation at target genes. We will continue to screen stable fly field submissions for presence of the kdr-his allele, and intend to expand the screening panel to any newly identified polymorphisms. The nationwide stable fly resistance monitoring efforts will continue and be coordinated by FL. All members of the multi-state project will be invited to participate, as will others not officially affiliated with the project. Our efforts will consist of nearly year-round surveillance, as stable fly populations shift from winter activity in the southern U.S. to a peak in the northern states and southern Canada in July and August.

b. Leveraging the Stomoxys and Musca genomes for novel control measures

Accomplishments during current Multistate Project. Both the house fly (Scott et al. 2014) and stable fly genomes were sequenced. The house fly genome is 691 Mb and contains a rich resource of novel protein coding genes, a high amount of repetitive elements, and substantial increases in copy number and diversity of both the recognition and effector components of the immune system, consistent with life in a pathogen-rich environment. There are 146 P450 genes, representing a significant increase relative to D. melanogaster and suggesting the presence of enhanced detoxification in house flies. Relative to D. melanogaster, house fly has also evolved an expanded repertoire of chemoreceptors and odorant binding proteins, many associated with gustation. The stable fly genome sequencing project is complete and gene family expansions were observed for cytochrome P450s, as well as immune system and chemosensory pathway genes. The availability of the sequenced genomes will accelerate numerous investigations into these important pests, especially those involving insecticide resistance.

Planned research for next multistate project. Having sequenced genomes allows for the identification of the mutations responsible for resistance at high resolution. We will evaluate a

new strain of house fly that cannot be killed by massive doses of permethrin. Using a new technique called bulk segregant analysis we will map the loci responsible for resistance, identify the genes at this locus, and run validation assays to determine which identified mutations are responsible for resistance.

Objective 3. Investigation of the microbial ecology, epithelial immunity, and vector competence of biting and nuisance flies

a. Identification of the key bacterial strains and their metabolites playing a major role in oviposition and larval development of stable flies (USDA-NE)
b. Investigation of the innate immune response of filth flies (KS, USDA-KS, MA)
c. Consequences of fly-bacteria interactions: selection effects and evolutionary outcomes (USDA-KS, TX)
d. Animal and human pathogen acquisition, dispersal and deposition by muscid flies (NC, MA, KS, USDA-KS, TN, PA)

a. Identification of key microflora and their metabolites playing a major role in oviposition and larval development of stable flies

Identification of common microbial communities in diverse larval habitats of stable flies. Stable fly larvae develop in diverse environmental substrates ranging from soiled animal bedding, hay residue, seaweed, mayfly carcasses, horse manure, and numerous crop residues including pineapple, beets, and sugarcane. The development of stable flies in crop residues has become especially problematic for countries such as Costa Rica, Australia, and Brazil where relatively new cropping methods have resulted in an explosive population of stable flies (Taylor in press). In the US, the economic threshold is reached when two or more stable flies are on both front legs of one animal. In Australia and Costa Rica, producers are encountering over 2,000 stable flies per animal. The typical description of larval habitat has been moist, decaying vegetation with the presumption that larvae are feeding on microbial communities associated with fermentation. However, new information out of Costa Rica and Australia indicate that gravid females may oviposit on freshly cut crop residue within minutes of harvest or even live plants. Clearly, more information is needed to assess the role of microorganisms in stable fly larval development. Identification of key bacterial groups involved in larval development may lead to the development of novel fly control strategies.

Illumina sequencing will be used to identify common microbial communities in diverse larval habitats of stable flies. Deep sequencing has been used to identify common microbial communities in horse manure, hay residue, lab media used to maintain lab colonies, and calf bedding. Samples from other types of developmental sites in Nebraska will be added including lagoon waste, corn silage, and alfalfa silage. Cooperative research agreements established with researchers in Costa Rica and Australia will allow for the inclusion of pineapple, carrot, celery, and other crop residues.

Identification and characterization of endosymbionts including Herpetomonas. The role of endosymbionts in host physiology and behavior is a rapidly developing branch of science. In many cases, the host is dependent upon its endosymbiotic community for resources. A recent survey demonstrated that trypanosomatids parasitize many species of flies. Best known are the

dixenous etiological agents of African sleeping sickness and n'gana (*Trypanosoma brucei*) and leishmaniasis (*Leishmania* spp.). However, in addition to these pathogens, many monoxenous trypanosomatids are symbionts in the guts of insects. Trypanosomatids have been identified and characterized in adults of several flies. However, until the recent observations of Friesen (unpublished) they were not known from stable flies. Representatives of these symbiotic groups were first observed infecting stable flies during a preliminary survey in southeastern Nebraska. Nothing is known of their taxonomy, life history, or most importantly, effects on their hosts. Each has the potential for development into a fly control technology.

Minimum infection rates of stable fly larvae infected with trypanosomatids will be quantified from pools of surface sterilized larvae that are homogenized and inoculated into liver infusion tryptose media. Presence or absence of trypanosomatids are assessed at 2-3 day intervals up to 14 days post inoculation by microscopic examination of cultures. Minimum infection rates are calculated with the PooledInfRate program. DNA will be isolated from cultures of trypanosomatids, sequenced, and compared to published GenBank sequences for identification. Anatomical location and extent of symbiont colonization within the fly will be visualized with histological and microscopic techniques.

b. Investigation of the innate immune response of filth flies

Differential utilization of dung as a developmental substrate for Muscid larvae. Flies in the subfamily Muscinae (Diptera:Muscidae), including the Stomoxyini (stable flies and horn flies) and Muscini (house flies and face flies), are important pests of livestock that impact production, weight gain and food safety, ultimately resulting in economic losses to producers (Harris et al. 1987, Jonsson and Mayer 1999, Meerburg et al. 2007, Taylor et al. 2012).While the biology of adults differs across these flies, larvae all four species require microbial communities for development (Hollis et al. 1985, Schmidtmann and Martin 1992, Zurek et al. 2000, Perotti et al. 2001, Albuquerque and Zurek 2014). Although larvae utilize and alter the microbial community of their respective developmental substrates, it is unclear whether the pathways regulating this interaction are a reflection of phylogenetic relationships or microbial utilization requirements. Differences in developmental substrate utilization may be due to variability in the ability to effectively utilize microbes. Bacteria consumed by larvae are digested by the synergistic activity of digestive enzymes (e.g. proteases, lipases) and anti-bacterial effector molecules produced by gut epithelia (Lemos and Terra 1991, Ito et al. 1995, Terra and Ferreira 1994, Nayduch and Joyner 2013). These immune effector molecules, such as antimicrobial peptides (AMPs), lectins, lysozymes, and reactive oxygen species, are components of the insect innate immune response and can be upregulated during fly-bacteria interactions in both adults and larvae, as shown in stable and house flies (Munks et al. 2001, Nayduch and Joyner 2013, Nayduch et al. 2013). These effectors may serve a dual-purpose in larval filth flies: digesting and eliminating bacteria in the gut while concurrently protecting a host from infection and invasion.

A cross-species comparison as it relates to common rearing conditions and utilization of microbes in the substrate is lacking. We propose to use transcriptome and microbiome analyses to investigate how four species of representative Stomoxyini and Muscini larvae utilize and alter the microbial community of a single substrate (dung) commonly used among these species. We will use a transcriptomic approach coupled with microbiome profiling (both 16S and 18S) to

capture “snapshots” of the larval genetic response and the associated bacterial abundance and diversity within the larvae and its corresponding larval substrate (dung) during the L2 and L3 larval stages of both species. Using this approach, we aim to determine whether larval transcriptome profiles are shaped by evolutionary relationships and/or by the nutritional utilization of the bacterial community.

Identification of antimicrobial peptides in the salivary glands of adult house fly when challenged with fungal spores and evaluating their effectiveness in destroying various pathogens. House fly adults are able to survive those nasty pathogens they ingest because of the various strategies they have evolved to destroy them before they kill themselves. One, and possibly the most important, strategy is their evolution of very effective suite of antimicrobial peptides that kill numerous ingested pathogens that are initially stored within the crop. One researcher has called the crop of adult flies a “sterilization organ.” It makes sense to eliminate many pathogens that might kill the fly prior to them entering the midgut with the ingested diet. We are working with the center for Mass Spectroscopy to identify various AMP found within the salivary glands of adult house fly when exposed to fungal spores. We are processing salivary glands from non-challenged flies and comparing the results with those of flies exposed to fungal spores. When a fly encounters a food source, especially if it is dry, it salivates. The saliva performs two functions: first, it liquefies the diet for ease of ingestion and secondly it produces AMP that enter the crop with the consumed diet. Within the crop, these peptides should be able to control the numbers and types of pathogens that enter the midgut. If we can interfere or better understand the involvement of these peptides and evaluate what happens within the crop, it might be possible to increase the effectiveness of those pathogens that kill the fly.

c. Consequences of fly-bacteria interactions: selection effects and evolutionary outcomes

Using genomic approaches to study fly-bacteria interactions. Studying the interactions between bacteria and filth flies is informative for control of nuisance and biting flies, and necessary for understanding how flies spread pathogenic bacteria. Laboratory experiments combined with genomic approaches are a powerful way to investigate fly-bacteria interactions. For example, metagenomic sequencing can identify the bacterial species and strains associated with the surface and digestive tracts of flies (Zheng et al. 2013; Singh et al. 2015). In addition, identification of genes that encode components of the immune system is essential for determining how flies naturally control bacterial proliferation. Analysis of the sequenced house fly genome revealed a massive expansion of immune genes, likely as an adaptation to the septic environment in which the flies live (Scott et al. 2014). Studying gene expression in filth flies infected with bacteria using genomic approaches (e.g., RNA-Seq) can reveal key immune genes that respond to bacterial infection (Sackton et al. 2017). Furthermore, experiments that allow bacteria to adapt to digestive tracts have the potential to identify key regulators of bacterial proliferation in the bacterial genomes (Sousa et al. 2017). Finally, bacteria can affect the behavior and development of flies. Molecules produced by *Proteus* modify blow fly behavior (Ma et al. 2012, Tomberlin et al. 2012, Liu et al. 2016, Yuan et al. 2016). Bacteria also affect the developmental rate of flies, but the specific mechanism by which this occurs is not clear (Crooks et al., 2016). Intriguingly, our unpublished work shows that fast and slow developing blow flies exhibit different sensitivities to rapamycin, an immunosuppressant (Chakrabarti et al. 2012). This suggests a link between the immune system and larval development. Genome sequencing and functional

genomics experiments have the potential identify the specific bacterial and fly genes that regulate these interactions.

Features of fly-associated bacteria. Genetic and phenotypic traits of bacteria isolated from blow flies and their environments are being characterized. We have surveyed flies associated with carrion-feeding insects (Zheng et al. 2013, Singh et al. 2015). The lab has used this information to pursue interactions of interest. For example, the lab conducted a number of experiments studying *Proteus mirabilis* interactions with *Lucilia sericata* (Ma et al. 2012, Tomberlin et al. 2012, Liu et al. 2016, Yuan et al. 2016), including the role of the microbe in chemical attraction of the fly and whether the genome sequence of such a strain is typical of other *Proteus mirabilis*. In addition, fly traits often depend on temperature. Likewise, all bacteria exhibit thermal performance variation. Therefore, it is possible that certain bacteria may exert greater impacts on flies at different temperatures than others. We propose to perform thermal performance assays for bacteria derived from blow flies and blow fly habitats (such as those in Yuan et al. 2016 and Yuan et al. 2017). Depending on the results of these experiments, species-specific profiling of bacteria will be done to determine how differences in temperature impact bacterial impacts on flies. For example, if two urease producing bacteria are associated with a blow fly, but one exhibits highest growth at 29°C while the other exhibits highest growth at 35°C, then urea related stress will be tested in flies across these temperatures when exposed to neither, one, or both bacteria. It will also be possible to compare fly-derived performance curves to similar curves for non-fly associated strains (ex. Does *Salmonella* from isolated from flies exhibit a different thermal performance curve than human or chicken associated strains?). Finally, we will profile microbial communities at different temperatures to determine if certain taxa accumulate in flies at different temperatures. Results from such experiments may identify when specific bacteria are expected to impact fly, human, and animal biology.

d. Animal and human pathogen acquisition, dispersal, and deposition by house flies

Transmission of pathogens that affect humans and poultry in Pennsylvania poultry facilities. Flies can serve as a vector to spread foodborne pathogens onto crops from adjacent lands. Farms growing fresh produce may not have livestock on the property but there are often animals, such as cattle and wildlife, nearby. Cattle and wildlife have been known to be reservoirs for Shiga-toxigenic *Escherichia coli* (STEC) and *Salmonella*. Since cattle operations may be in close proximity to farms that are growing fresh produce, it could allow insects to transfer these pathogens to the crops. Talley et al. (2003) observed a high presence of filth flies, belonging to the *Muscidae* and *Calliphoridae* families, at a single spinach production site, which had a 61% *E. coli* O157:H7 contamination rate. Filth flies can transfer *E. coli* O157:H7 in their regurgitation spots onto spinach, in which bacteria have been observed to grow on the crop surface (Talley et al. 2003, Wasala et al. 2013). Additionally, STEC has also been shown to survive on filth flies for up to 13 days after contamination (Talley et al. 2013). The impact vectors play in produce contamination is not fully understood. Data characterizing the prevalence of foodborne pathogens on integrated farms farm fly populations is lacking. The prevalence of STEC and *Salmonella* associated with various genera of flies sampled/collected on a farm with both produce and beef cattle has been investigated. Flies were collected from trap locations placed throughout integrated farms with low-density beef cattle as well as fruit and vegetable plots. Our preliminary data suggest that *Muscidae, Calliphoridae,* and *Sarcophagidae* spp. are more likely

to carry *Salmonella* and Shiga-toxigenic *Escherichia coli*. Transmission from flies can occur from physical transfer, regurgitation and excreta onto produce (Talley et al. 2013, Kobayashi et al. 1999). Further research is needed to determine the impact of cattle density, produce type and farm size as well as interventions that may alter fly patterns to reduce the risk of produce contamination.

Pathogens transmitted mechanically by house flies in poultry facilities are a concern. Both human and animal pathogens may be found in these facilities, and can cause losses in production and worker productivity, and even bird mortalities. The transmission mechanism of these pathogens between birds and/or facilities is not always clear. Pathogens such as *Campylobacter*, *Clostridium perfringens*, and avian influenza, can be present in facilities with high levels of biosecurity, leaving questions on pathogen origin. Recently, the ability of house flies to transmit pathogens of interest affecting poultry has been questioned. The goal of this project is to identify the occurrence of common pathogens affecting poultry on field collected house flies, and identify the transmission competence of these flies.

Role of house flies and face flies in transmission of pathogens associated with Bovine Respiratory Disease in cattle feedlots. Bovine respiratory disease (BRD) has a multifactorial etiology and develops as a result of complex interactions between environmental factors, host factors, and pathogens. Environmental factors (e.g, weaning, transport, commingling, crowding, inclement weather, dust, and inadequate ventilation) serve as stressors that adversely affect the immune and nonimmune defense mechanisms of the host. In addition, certain environmental factors (e.g, crowding and inadequate ventilation) can enhance the transmission of infectious agents among animals. Several infectious agents have been associated with BRD. An initial pathogen is typically the BRD virus that alters the animal defense mechanisms, allowing colonization of the lower respiratory tract by bacteria such as *M. haemolytica, P. multocida,* and *H. somni.* House flies are a potential mechanical and/or biological vector for these bacteria among sick cattle and from sick to healthy cattle; however, this role has not been investigated so far. We propose to: a) optimize culturing and PCR-based detection techniques for *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* in house flies; b) evaluate the prevalence *M. haemolytica, P. multocida,* and *H. somni* in house flies and face flies collected from sick pens in commercial cattle feedlots, and c) evaluate the prevalence of *M. haemolytica, P. multocida,* and *H. somni* in house flies and face flies collected from the outside of sick pens in commercial feedlots.

Acquisition and transmission of Salmonella between house flies when exposed to cantaloupe *Salmonella enterica* ser. Typhimurium. (*S.* Typhimurium) is a pathogen harbored by livestock that causes food-borne illnesses. In agricultural settings, house flies acquire *S.* Typhimurium by developing in and feeding upon manure from infected animals. As adults, house flies can serve as a bridge between unsanitary and sanitary environments. We have previously demonstrated that both male and female adult house flies acquire and harbor *S.* Typhimurium after exposure to inoculated cattle manure (Thomson et al. 2017). However, fly to fly transmission of the bacterium, after acquisition, has not been demonstrated. *Salmonella* spp. can contaminate produce such as cantaloupe, where they proliferate and persist. Because house flies are attracted to and feed upon human food items, we propose to investigate the transmission of *S.* Typhimurium from infected flies to cantaloupe, from inoculated cantaloupe to flies, and between

infected to uninfected flies in the presence and absence of cantaloupe. We hypothesize that *S.* Typhimurium will survive in house flies and that the amount transferred from *Salmonella* flies (SF) to control flies (CF) will increase over time regardless of whether cantaloupe is present or absent. Furthermore, we predict that the transmission of *Salmonella* from food-fly, from fly-food, or from fly-food-fly will increase over time. Adult female house flies (mated, 5-7 days old) will be given *ad libitum* sugar water and will be exposed to manure inoculated with sterile PBS (CF) or *S.* Typhimurium (SF) for 12 h. To test for survival of *S.* Typhimurium, the SF will be placed individually in empty jars. To test for transmission of S. Typhimurium, the flies will be placed into jars containing either (1) a single SF with fresh cantaloupe (fly to food transmission), (2) four CF with *S.* Typhimurium-inoculated cantaloupe (food to fly transmission), or (3) a single SF with four CF with or without fresh cantaloupe (fly to fly transmission, with or without food). Our results will determine if flies can successfully transfer *S.* Typhimurium to, and become infected from, cantaloupe. In addition, this project will determine if the presence of cantaloupe facilitates fly to fly transmission of *Salmonella*. Understanding the dynamics of fly bacterial transmission between other flies and food can help in determining potential health and food safety risks.

Modulatory and controlling factors affecting crop contractions in flies. The diverticulated crop of adult flies is unique in the insect world (Stoffolano and Haselton 2013). It provides the fly with an opportunity to imbibe a meal when encountered in environments where food is usually randomly distributed. Because many of these flies frequent habitats of filth and, also the fact that they eat from these same environments, they normally ingest human and domestic animal pathogens, which initially enter the diverticulated crop. Based on the meal and crop volume these same flies, when visiting either food service areas, field crops, or other domestic situations, regurgitate the crop contents, which also contains numerous pathogens. Using morphological (SEM, TEM, AND CONFOCAL microscopy) and electrophysiological techniques, a more detailed examination of the crop lobes and duct of various flies is being conducted. Already, a new and novel enteric group of neurons has been identified in the crop duct nerve bundle and a search for their role in crop regulation, especially regurgitation, is ongoing. At the same time, collaboration with a group of Italian researchers has evaluated and discovered various modulatory neuropeptides and biogenic amines that are involved in crop modulation. We are tackling the important question of the importance of the gut/brain interaction. If we better understood the control mechanisms regulating crop control, both intake filling and emptying via regurgitation, there might be other ways to kill flies by interfering with food intake and regurgitation from this important organ that are different from traditional insecticides.

House fly as a vector of various pathogens – role of the diverticulated crop. House fly adults are able to survive those nasty pathogens they ingest because of the various strategies they have evolved to destroy them before they kill themselves. Because of their environment, which consists of walking through and imbibing various substances, which often include fluids of animals that contain numerous pathogens. It is important to know how these pathogens are acquired, where they go in the fly, and how they are transmitted. Our laboratory is involved in investigating various pathogens such as *E. coli, Chlamydia trachomatis*, and *Vibrio cholera* as to how they are taken into the crop and what happens to them following uptake.

Flies as vectors of pinkeye and mastitis in cattle. Two diseases continue to impact dairy systems throughout the US: infectious bovine keratoconjunctivitis (IBK or pinkeye) and mastitis. Both diseases occur in cattle of all ages but are damaging in calves and heifers. Clinical signs of IBK are excessive lacrimation, inflammation of the eye, conjunctival edema, corneal opacity, and ulceration (Postma et al. 2007, Alexander 2010). Infection of IBK causes weight loss, impaired vision, eye disfigurement and blindness. IBK is a common but preventable eye disease of cattle caused by the bacterium *Moraxella bovis*, a Gram-negative rod that carries genes for the expression of pilin and cytotoxin allowing for attachment to the eye and erosion of the cornea, respectively. Autogenous vaccines targeting pilin attachment to the eye are common and serve as a key component to management of this disease. Unfortunately, vaccine efficacy has been inconsistent (Cullen et al. 2017) making the goal face fly (*Musca autumnalis*) management an on-going concern of the producer.

Mastitis is characterized as an inflammation of the mammary gland caused by bacteria manifested in poor milk quality, an inflamed and swollen mammary gland (Olde Riekerink et al. 2008, Oliver et al. 2004). Subclinical mastitis is usually monitored by measuring the somatic cell concentrations (SCC) of leucocytes in milk. Somatic cell counts increase in response to a bacterial infection of the mammary gland. Traditionally heifers were thought to be at a low risk for mastitis (Fox 2009). Clinical and subclinical mastitis have been reported as a significant problem in primiparous dairy cattle with a higher prevalence and incidence in heifers than cows, especially early in lactation (McDougall et al. 2009). Horn flies (*Haematobia irritans*) play a significant role in the occurrence of mastitis in dairy cattle (Oliver et al., 2004), fly control is an essential component of mastitis control (De Vliegher et al. 2012). Studies have linked fly induced teat damage to mastitis risk among heifers (Owens et al. 1998).

Common causes of bacterial infection of the cow's udder include *Staphylococcus aureus*, *Streptococcus uberis*, *Escherichia coli* and *Enterococcus* species (Godden et al. 2003). The horn fly supports sufficient *S. aureus* to infect cattle while feeding on teats and there is epidemiologic evidence that fly burdens and feeding may impact pathogen spread in dairy herds (Anderson et al. 2012). The face fly harbors and transmits *Moraxella bovis*, the causative agent of pinkeye in cattle (Glass et al. 1982). These flies are clearly linked to the spread of some cattle diseases, their activities impact cattle welfare and productivity, and fly control is a critical issue for the wellbeing of cattle.

Our goal is to evaluate the impact of integrated fly management strategies in dairy systems, and to determine if these strategies reduce fly abundance and occurrence of mastitis in dairy herds in a cost-effective manner. We will be monitoring fly populations, and tracking fly-borne infections in their hosts, and monitoring somatic cell counts as indicators of mastitis. Bacteriological studies will focus on the quantification and genotyping of disease specific bacteria and the ability of these pathogens to be carried by and persist in and/or on flies.

Objective 4: Characterize population biology of biting and nuisance flies

a. Characterize effects of climate and landscape features on dispersal (KS, TX, USDA-NE)
b. Phenology of biting and nuisance flies (FL, KS, TN, USDA-NE)
c. Genetic structure of biting and nuisance fly populations (TN, TX, USDA-NE)

Population biology deals with the growth and regulation of populations, their structure, both age and genetic, and their interactions, both with each other and with the environment. Flies become economically important problems only when their populations reach excessive level. In this objective, the dynamics of pestiferous fly populations will be studies with the goal of developing methods to predict changes in population levels on local and regional levels. Population levels are dependent upon reproduction, mortality, immigration and emigration.

a. Characterize effects of climate and landscape features on dispersal

Dispersal, especially long range dispersal, can be difficult to document or quantify (Nathan 2001), but is exceedingly important for understanding population dynamics and developing management plans. Multiple methods can be used to evaluate dispersal in the field (Nathan et al. 2003). Two methods appropriate for characterizing fly dispersal are Eulerian (mark recapture) and genetic structure. A third method for evaluating dispersal potential in the laboratory is the use of a flight treadmill. All have been applied to nuisance flies; however, data on dispersal distances, phenology, and ubiquity of dispersal remain elusive.

Laboratory studies with flight treadmills indicate stable flies are capable of flying up to 29 km in 24 h (Bailey et al. 1973). Horn flies were capable of flights of 5km in Georgia to 11.74km distances in New Mexico (Sheppard 1994, Kinzer and Reeves 1974). Horn flies, although dependent on the host for sustenance, survived up to 10 hours off of a host (Kinzer and Reeves 1974).

In the field, stable flies were observed to disperse 8 km in <2 h in south-central Oregon (Eddy et al. 1962) and up to 225 km over several days in the Florida panhandle (Hogsette and Ruff 1985). Gersabeck and Merritt (1985) found that 50% of flies released on Mackinac Island, MI, were recaptured within 0.45 km, and 90% were recaptured within 1.65 km. Flies released close to horses dispersed less than those released further away, and none of the released flies were collected on the Michigan mainland, 11 km away. Todd (1964) found that dairies adjacent to fly development sites in New Zealand were heavily infested, whereas stable flies were “no problem” within 1.6 km from developmental sites. More recently, studies at a mixed agricultural site in southeastern Nebraska observed that 50% of stable flies dispersed more than 1.6 km from their larval developmental sites and 5% dispersed more than 5.1 km (Taylor et al. 2010). In Florida, stable flies were observed moving at least 1.5 km within 48 hours from blood feeding sites to resting and / or oviposition sites (Pitzer et al. 2011) while in Belgium they dispersed a maximum of 300 meters (Lempereur et al. 2018).

Horn flies can disperse up to 5 km in less than 2 hours when released distant from potential hosts (Sheppard 1994). In studies done under the auspices of our previous Multi-state project in North Carolina, small groups of young stock on 1-2 hectare pastures were used in a mark and recapture study. Horn flies were marked with fluorescent dyes and released. Horn flies were able to locate cattle 300 to 1100 meters away in as little as 3 minutes, but often it took longer, 21and 38 minutes for the first fly to arrive. At wind speeds of 10 km/h, 1.3% of the flies located the cattle by flying against the wind, but when wind speeds were 7 km/h, significantly more, 3.5% of the released flies, located cattle by flying against the wind. At this small scale, push pull was expected to be effective because abandoning flies have alternate hosts in close proximity. To

study this one group of cattle was treated with 1% geraniol mixed in mineral oil and the horn flies were observed to abandon the cattle or if hit with the spray fall to the ground. After the treatment, marked flies were released in the pasture with treated cattle. Some marked flies landed on body parts where the spray missed but most dispersed from the pasture seeking other cattle. The marked flies arrived at the untreated cattle pasture within 7 minutes. Of the total number of flies released, 8% traveled to the untreated cattle, 227 m distant. It is unknown if the remaining marked flies arrived later or dispersed elsewhere. Mark and recapture studies will further our understanding of spatial limits for flies to acquire new or alternative hosts.

Nuisance flies can readily disperse long distances, but appear to do so only when resources, either hosts or oviposition sites, are inadequate (Hogsette and Ruff 1985, Taylor et al. 2010). Relationships between weather phenomena, landscape features, and phenology on dispersal remain unknown as do the cues, mechanisms, and extent of long range dispersal.

Mark-recapture. As part of our efforts to expanding Push-Pull Strategies to a broader assemblage of pasture pests, horn fly, face fly and stable fly, mark and recapture studies will continue in NC to examine dispersal distances of horn flies forced to abandon repellent-treated cattle. Topography and vegetative barriers likely have a role in the successful dispersal and acquisition of a new host by flies (Fried et al. 2005). Horn flies will be collected from cattle, marked with fluorescent powders and release in the vicinity of repellent-treated cattle. Untreated herds located at distances of 1 to 4 km will serve as recipient animals. Placement of recipient herds with careful attention to topography and barriers will provide information on the probability of flies acquiring hosts under such conditions. Collected horn flies from the recipient animals will be examined for color markers to establish measurable dispersal distances. A similar analysis was applied to stable fly dispersal (Taylor et al. 2010) using the Turchin and Thoeny (1993) model. Fly captures from distances of 1, 2, and 4 km will be calculated as a radial distance from the release site and analyzed using an empirical regression model to examine rates of decline in the daily catch rate with days after release and distance. Slopes for distance by day will be compared using analysis of covariance (Taylor et al. 2010).

Flight Mill Studies. To assess the flight capacity of horn flies and other pasture flies we will use a flight mill to establish flight distances, flight speed and the duration of flight (Nilssen and Anderson 1995). Proof of concept experiments in NC using wild caught horn flies we demonstrated a maximum flight of 8.2 km before the insect died. These experiments will be expanded to include colony reared insects of known ages, sex and nutrition and with comparisons to wild-type flies. Addition evaluations will include face flies, house flies and stable flies secured from USDA colonies.

b. Phenology of biting and nuisance flies

Stable fly larvae are capable of developing on a wide variety of substrates (Hogsette et al. 1987, Cook et al. 2017). Although temperature dependent growth tables for stable fly were developed during previous projects (Lysyk 1998, Gilles et al. 2005). Interactions between temperature, and substrate, including microbial associations, need to be addressed. Preliminary studies initiated in the previous multi-state project found that diet quality had little effect on stable fly rate of development when temperatures were between 20 and 30° C. However, when rearing

temperatures were either below or above that range, stable flies in low quality substrates developed faster than those in higher quality substrates. Flies reared on low quality substrates were smaller than flies developing in higher quality substrates at temperatures of 25° C or less. However at higher temperatures, substrate quality had little effect on adult size.

Investigations of interactions between developmental substrates and temperature on life history parameters will continue with the addition of metagenomic analyses to assess the microbial communities associated with various substrates. Substrate quality will be adjusted by diluting standard laboratory diets with inert bulking materials, vermiculite or sand. In initial studies, nutrients, fish meal and wheat bran, will be reduced. Life history parameters, developmental time, survival, and size for stable flies developing in substrates with 1/8, ¼, ½, and standard nutrient concentrations at 15, 20, 25, 30, and 35° C will be compared. Pupal weight and length of the discal medial cell of the wing will be used to access size. One hundred fifty stable fly eggs will be placed on 100 g of media in 150 ml cups for each experimental unit. Each substrate-temperature treatment will be replicated three times. Cups will be examined daily and all pupae will be removed and placed individually in wells of 48 well plates. Pupae will be weighed the same day they were collected. Date of pupation, date of adult emergence, sex, and length of the dm cell will be recorded for each adult. Data will be analyzed with general linear mixed models. Microbial communities associated with each substrate-temperature treatment will be assessed with metagenomics analyses of 16s and 18s rRNAs. Similar analyses will be conducted with substrates with varying ratios of starch and protein components and using natural substrates. Lengths of the dm cell of field collected flies will be compared to those of the experimental flies to gain insight into the developmental substrates and conditions of the field collected flies. Associations of microbial communities on substrate quality will be evaluated.

c. Genetic structure of biting and nuisance fly populations

Biting midges. Although population genetic studies have been done with several nuisance flies, they have been limited by the number of insects and variable loci available for analysis. Genomic and high throughput technologies have reduced costs and increased access to variable genetic loci. Application of these technologies may increase the resolution of genetic analyses. *Culicoides sonorensis*, a biting midge, is reported from British Columbia to Central Mexico, east to the Mississippi river and occasionally in the Mid-Atlantic and Gulf-Coast states (Wirth and Jones 1957, Holbrook et al. 2000). This species is the main vector of the disease-causing pathogens of Bluetongue (BT) and Epizootic Hemorrhagic Disease (EHD) in North America, and cost the U.S. an estimated $130 million annually (Stelljes and Barry 1999). These diseases affect managed cows, sheep, and deer, as well as many wild ruminants. Though BT and EHD can be fatal, even after surviving contraction, the animal may develop chronic health problems and lameness as a result (Mellor et al. 2000). Despite the ecological and economic importance of *C. sonorensis*, its ecology and distribution remain unclear.

Population genetic studies of other species of *Culicoides* have been carried out in Europe, Africa, and Australia. Using microsatellite and sequencing data, many have found a high degree of gene flow between populations and determined that wind-mediated dispersal is a main factor in their distribution (Onyango et al. 2015, Jacquet et al. 2016, Tay et al. 2016). The goal of this project is to compare populations of *Culicoides sonorensis* across North America in order to measure gene

flow and detect dispersal patterns. The proposed project will identify single nucleotide polymorphisms (SNPs) from individuals and use them to determine population structure. The primary deliverable will be a population genetic analysis of *C. sonorensis* and an assessment of the relationships between identified populations.

In our study, we will investigate the population dynamics of the main pathogen vector of hemorrhagic disease in North America. DNA will be extracted from individual C. sonorensis and sequenced using next generation technology. Sequence reads will be assembled and relevant SNPs will be used in high throughput genotyping, aided by access to the C. sonorensis reference genome. We will assess the genetic composition of identified population as it relates to dispersal patterns, hybridization, vector capacity, and gene flow. Through an extensive sampling effort by members of this multi-state project, we will clarify the North American distribution of C. sonorensis, gain insight into its ecological interactions, and conduct disease outbreak risk assessments by tracking vector dispersal patterns.

Midges collected from North America will be identified and cataloged based on species identification and geographic location. DNA will be extracted from individual C. sonorensis and sequenced using a double digest restriction associated DNA (ddRAD) protocol. Sequence reads will be assembled and relevant SNPs will be used in high throughput genotyping, aided by access to the C. sonorensis reference genome. Relevant SNP's will be used in population genetic analysis.

A sufficient amount of DNA may not be obtainable from single individual midges. In this case, whole genome amplification will be performed on samples yielding low amounts of DNA. Samples from the same geographic location may have to be pooled if the first two methods fail. If samples must be pooled, the ability to identify hybrids within populations would be lost. The use of whole genome amplification before ddRAD seq has the potential of adding false variation.

Tabanids. Classification, identification, and species delimitation in some groups of nuisance flies can be difficult due to ambiguous morphological characters. Of special note, are the horse flies or Tabanidae. Markings on the dorsum of the abdomen are used to differentiate many of the species in the genus *Tabanus*. Unfortunately, these markings can vary significantly within species and their appearance can change depending upon the angle of view and lighting. Furthermore, immature horse flies are difficult to rear and larvae of many species have not been properly associated with their adults (Iranpour et al. 2004). The application of molecular systematic techniques will provide improved characters for the identification and classification of flies in this difficult group.

To improve identification and characterize the population biology of Tabanids, Trout-Fryxell, Mullens, and Kline are collaborating with members of the Regional Hatch project to describe the morphology, genetics, phenology, and ecological habitats of Tabanids. Specifically, they will begin working with Tabanus east of the Mississippi and describe members of the suspected Tabanus sulcifrons complex. They will use a combination of traditional taxonomy, phylogenetics, ecological niche modeling, quantitative morphometrics, scanning electron microscopy, and spectrometry to add to the biological data on geographic and seasonal distributions of this species-rich group. Samples will be collected and/or assessed with a variety

of methods including traditional trapping, use of citizen scientists, and museum collections, and then incorporated into the project. Resulting data will lead to improved tabanid identification and an increased understanding for a taxonomically diverse group.

Stable flies. Population genetics has been used as an indirect measure of dispersal, and this has been true for stable flies. Allozyme studies in northern Florida implicated inland livestock facilities as sources of stable flies appearing on coastal beaches (Jones et al. 1991). Several studies using allozyme, AFLP, microsatellite, and mitochondrial markers found stable fly populations exhibited low levels of differentiation indicative of high levels of gene flow / dispersal (Ascunce et al. 2009; Jones et al. 1991; Kneeland et al. 2013; Krafsur 1993; Marquez et al. 2007; Szalanski et al. 1996). Physical markers such as pollen (Jarzen et al. 2008), and blood meals (Pitzer et al. 2011) were used to document stable fly movement as well.

With the decreased costs and inputs into the genomic sequencing techniques and increased chances to identify variation (Depristo et al. 2011), Friesen and Trout Fryxell will collaborate with other members of the regional hatch project to identify phenotypic and geographic distinct SNPs in North American stable flies and conduct a population genetics study using the provided samples. We will use next-generation sequencing approaches (genome-by-sequencing) for SNP discovery. The current collection consists of male and female flies from eight locations, representing two phenotypes. The discovered SNPs will be analyzed in a preliminary population genetics study to determine the population structure of stable flies in North America. Resulting data will lead to further studies on dispersal.

Single Nucleotide Polymorphisms (SNP) will be identified in stable fly using genomic tools. Once an adequate number of SNP have been characterized, high through put methods will be used to score SNP genotypes of stable flies from 60 wide spread populations within the United States and among samples collected world-wide to evaluate population structure. Temporally repeated collections (5 / yr) from 9 sites representing north-south (Minnesota-Texas) and east-west (North Carolina-Oklahoma) transects will be analyzed to evaluate dispersal among populations. Stable flies from 5 populations will be classified into phenotypic classes (host preference and larval developmental sites). Genetic analysis of flies relative to class using efficient mixed-model association will be used to evaluate local adaptation / differentiation of stable flies relative to hosts and larval developmental habitats.

Objective 5: Extension and community engagement

a. Improve project website to maximize extension and community engagement
b. Demonstrate research value to stakeholders and funding decision-makers
c. Seek funding to support these extension/outreach efforts by developing proposals that will be submitted to various granting agencies including our Regional IPM Centers.

a. Improve project website to maximize extension and community engagement

The ability to extend new knowledge to those who can benefit from it is perhaps one of the most important aspects of a multistate project. By prioritizing extension as a project objective, the members of this multistate project are signaling their commit to collaboratively pursuing extension and community engagement related to the goals and outcomes of this project.

During the previous 5-year project (S-1060), project members identified a stakeholder need to have an online searchable pesticide database for control of arthropod pests of livestock. This database was developed by project members, and is now available to the public through the Insect Pest of Animals website (www.veterinaryentomology.org) developed by project members using funds available to the multistate project team following selection of the S-1060 project as the winner of the Excellence in Multistate Research Award by the National Experiment Station Directors. The Insect Pests of Animals website is a collaboratively-managed website for the membership of this multistate project.

As a main component of objective #5, members will expand our project website to increase our reach to stakeholders and decision makers, in particular providing information related to project goals and outcomes. In addition, website materials will support stakeholder education related to veterinary entomology. Initially, this will be accomplished by providing links to veterinary entomology extension websites hosted by our participating stations and by sharing content as is appropriate. During the first 1-2 years of this project, members will identify a more specific framework for collaborative extension of information. It is anticipated that our project website will eventually include documents, images, videos, and other content to support training and education of stakeholders related to pest management in animal agriculture.

By project end, we aim to develop our project website as a national repository of extension products that all project members will support and utilize for their extension programs. We will look at additional social media options, taking particular advantage of our younger and more technologically savvy members to identify opportunities to provide additional pest management education through social media. As examples, our members have been experimenting with producing pest surveillance and pest management videos placed on YouTube that can be linked to our website to provide stakeholder training. These training materials would be particularly useful as educational products aimed toward youth farming organizations like 4H and FFA.

b. Demonstrate research value to stakeholders and funding decision-makers

To garner the support of our livestock and poultry industries, we will focus efforts on assessing the economic impacts of pest flies to animal production. Using as an example a recent update of the economic impact of stable flies on cattle (Taylor et al. 2012), project members will evaluate current economic impacts of other important pest flies (e.g. horn flies, face flies, house flies). Economic impact reports describe the financial costs associated with the presence of pests when pest abundance breaches an economic threshold. These economic impact analyses are particularly meaningful to both producers and to funding decision-makers!

Project members will also review and update the Research and Extension Needs for IPM of Arthropods of Veterinary Importance document produced as a Proceedings of a USDA-sponsored workshop in 1994 (https://www.ars.usda.gov/southeast-area/gainesville-fl/center-for-medical-agricultural-and-veterinary-entomology/mosquito-and-fly-research/docs/lincoln-workshop/). This document was last updated in 2001 and is in critical need of reevaluation. As part of this effort, project members will work with the USDA Regional IPM Centers to develop "Pest Management Strategic Plans" for the pest flies addressed in this multistate project. In the process of developing these Strategic Plans, we will develop close partnerships with livestock

and poultry producers, animal scientists, agricultural engineers, and others whose work has an impact on (or is impacted by) livestock pests; developing these connections with key stakeholders will help to ensure rapid and timely transmission of information among all stakeholders across the US.

Extremely valuable livestock entomology research continues to be published and presented at scientific meetings, conferences, etc. It is essential that this research is converted into a form that can be used by extension personnel, veterinarians and policy makers across the US for immediate benefit to our stakeholders. To accomplish this, knowledge gained from the studies organized under the umbrella of this multistate project will be written up by project participants in a "public-ready" impact statement format for distribution to our stakeholders, including but not limited to conventional and organic livestock and poultry producers, veterinarians, commodity and industry organizations (e.g. Farm Bureau), state and federal legislators and regulators, newspapers, trade journals and magazines, TV, radio, etc. Impact statements will be disseminated through our project website, a project blog, and through more traditional communication directly to stakeholders and decision-makers. For decision-makers to fully understand the far-reaching, beneficial impact to animal agriculture that our current science offers, it is extremely important that our administrators, industry leaders, regulators, legislators, etc., at the state and national levels are made aware of the large number and extensive breadth of livestock entomology studies and extension programs currently being conducted by researchers at land grant universities and the USDA-ARS and by extension personnel across the US.

Previous research has not investigated livestock producers' willingness to adopt horn-fly management techniques into their programs; however, agricultural producer surveys are extensively used to assess technology adoption by providing values and perceptions. While it is established that flies are a significant pest of livestock, it is uncertain if producers recognize the importance of their control, how they can or should implement control, and if they value control options. The damage caused by flies to livestock, the increase in insecticide resistance, and evidence of economic and animal welfare concerns warrants a producer survey that determines producer awareness, value, current management and treatment options, preferences, and perceptions concerning fly control and management.

We will implement a cattle producer survey to assess cattle producers' (a) interested in fly control, (b) the value they would place on having flies controlled in their cattle herds, (c) how they value control and management, (d) how they would prefer to treat animals, and (e) producers ideas of the effects of veterinary pests on cattle production. We will begin by evaluating producer's perceptions of horn flies and proceed to include other veterinary pests as projects permit. This study allows us to survey producers for the economic value of horn fly control and control valued further down the production stream. Similar to previous studies, we expect farm size, financial considerations, perceptions of the management practice, control of outcomes, and risk to influence cattle producers' interest. We will begin with a Tennessee cattle producer survey to gauge if cattle producers are interested, value, how they value, their preferences, and their knowledge of the effects of veterinary pests on cattle production. We will target producers over the age of 18 and anticipate 500-1,000 cattle producers to respond to our survey. We will analyze survey results using descriptive statistics, correlation analysis, and regression analysis. We will determine how producer interest and value of the control varies by

farm size, farmer demographics, location, and other key variables. Results will provide information on producer potential to adopt novel technologies and the value producers place on management.

c. Seek funding to support these extension/outreach efforts by developing proposals that will be submitted to various granting agencies including our Regional IPM Centers, etc.

During our past 5-year project, we had support from the Western Region IPM Center to develop our pesticide database which formed the kernel of our new project website. This funding was critical to move our project down this very productive path for extending important project outcomes. We will continue to seek funding from regional IPM Centers and other agencies or funding opportunities that will support the collaborative efforts of our project members to expand and improve our extension and community engagement goals. In particular, funds are available from the IPM Centers to support development of the Pest Management Strategic Plans discussed in 5b above, and completion of these Strategic Plans will improve funding success of our project members by focusing research toward areas of greatest concern to producers. Project members will also seek funding as a component of their research-oriented grant proposals to support extension of their funded project outcomes through the multistate project website and through development of impact statements distributed through the multistate project. It is expected that funding for extension efforts is desirable or even necessary for inclusion in proposals to USDA-NIFA grant programs.