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To the Graduate Council:

I am submitting herewith a thesis written by Emily Luc entitled "The Horn Fly and its Association with Milk Yield, Milk Quality, and Staphylococcus Mastitis in Organic Dairy Herds." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Gina M. Pighetti, Major Professor

We have read this thesis and recommend its acceptance:

Rebecca T. Trout Fryxell, Liesel G. Schneider

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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

The Horn Fly and its Association with Milk Yield, Milk Quality, and *Staphylococcus* Mastitis on Organic Dairy Herds

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Emily Kathryn Luc

August 2020

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Abstract

Summer mastitis is a persistent challenge on dairy farms. Greater understanding of factors affecting mastitis will aid in developing management programs. Both mastitis and fly populations increase during summer, but the relationship between the two is unknown in lactating dairy cows housed on pasture. Our objectives were to 1) determine the association between fly numbers, somatic cell count (SCC), and milk yield on organic dairy herds and 2) identify specific *Staphylococcus* mastitis pathogens (*Staph. aureus*, *Staph. chromogenes*, *Staph. hyicus* and *Staph. agnetis*) in quarter milk samples and horn fly populations. Four USDA-certified organic herds located in Tennessee and Kentucky were enrolled in the study, with an average herd size of 55 lactating cows. Sampling occurred between May 2019 and October 2019. For the first objective, no relationship was observed between horn fly numbers and logSCC. Days in milk (DIM) had the most significant effect on the change in SCC. A significant negative relationship was found between horn fly numbers and milk weight, with a 0.99 kg decrease in milk yield per day with every 100 horn fly increase. An association was observed between a horn fly pool testing positive for *Staph. chromogenes* and both the individual cow and herd testing positive for the same bacteria. Sex of the fly pool and whether it was collected off the dorsal or ventral midline, had a significant association with type of bacteria present in the pool. Female horn flies were more frequently found with *Staph. aureus*, *Staph. chromogenes*, and *Staph. agnetis* compared to male horn flies. Compared to flies collected from the dorsal midline, horn flies collected off the ventral midline had a greater probability of carrying *Staph. aureus* and *Staph. agnetis,* and a trend was seen for *Staph. chromogenes*. With this information, it can be determined that reduced milk weight during the summer may partly be contributed to an increase in horn fly numbers. Common *Staph.* mastitis pathogens were also found in horn fly populations

and may be a source of transmission on farm. This study suggests the importance of horn fly control on dairy farms during the summer.

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CHAPTER I:

Staphylococcus Mastitis, Milk Yield, and Horn Flies in Organic Dairy Herds: A Review

Introduction

Mastitis, an inflammation of the mammary gland, is the most common disease found on dairy farms in the United States such that nearly all dairy producers reported having at least one case of clinical mastitis and about one fourth of all cows had clinical mastitis at some point in a single year (USDA, 2016). Mastitis is also the number one use for antibiotics on commercial dairy farms (Redding et al., 2019). Factors such as parity, stage of lactation, and season have an effect on mastitis. Older cows have a higher risk of developing mastitis than younger cows (Gröhn et al., 1995). Early stage of lactation is also associated with the development of clinical mastitis (Breen et al., 2009). Hogan et al (1989) found that clinical mastitis is highest during the summer months, while decreasing throughout the fall and winter months (Hogan et al., 1989). In a similar study, bulk tank somatic cell count (**BMSCC**) tended to increase during the summer and decrease in the winter (Cicconi-Hogan et al., 2013). When the probability of subclinical mastitis and isolated organisms was compared between the years of 2017 and 2018 on organic herds, cows were 2.15 times more likely to have subclinical mastitis in summer compared to fall, winter and spring (OR=2.15; 95% CI: 1.49, 3.10) (Luc et al, 2019). *Staphylococcus hyicus* was also associated with a higher probability in the summer, regardless of the year (Luc et al, 2019). Temperature and humidity increase during the summer, promoting bacterial growth (Smith et al., 1985). The temperature and humidity also cause heat stress resulting in immunosuppression of the cows (Aggarwal and Upadhyay, 2013). Understanding the effects summer has on mastitis is a crucial factor in controlling the disease on organic dairy herds. According to the NAHMS study in 2013, 87.3% of all mastitic cows in non-organic herds were treated with an antimicrobial (USDA, 2016). Organic dairy farms have the added challenge of treating and preventing mastitis

without the use of antibiotics or synthetic products (USDA, 2013). Prevention and understanding the risk factors associated with mastitis is key to controlling the disease in organic dairy herds.

Fly populations are known to increase during the summer, which is also when mastitis increases. Various fly populations can play a role in transmitting bacteria such as the face fly, house fly, stable fly and horn fly (Christensen, 1982). Horn flies carry *Staphylococcus aureus*, which has been found to cause summer mastitis in heifers (Nickerson et al., 1995). When samples were taken from flies, heifers and multiparous cows to compare the subtypes of *Staph. aureus*, all isolates from horn flies had identical DNA fingerprint patterns with majority of isolates coming from heifer samples. A common subtype was found in both heifer and fly isolates suggesting horn flies may play a role in the transmission of *Staph. aureus* to heifers (Gillespie et al., 1999). In a similar study, 55.8% of horn flies tested positive for *Staph. aureus;* whereas 13% and 17% tested positive for *Staph. aureus* in milk samples and heifer colostrum samples, respectively (Anderson et al., 2012). This suggest that flies and heifer body sites could be an important source of *Staph. aureus* for heifer intramammary infections. When fly control such as deltamethrin, is implemented on farms during peak fly season, a reduction in somatic cell count can be seen within 30 days of application. Cows not treated with fly control had an increase in somatic cell count from day 0 to 20 (Arsenopoulos et al., 2018). The use of fly control also reduced the amount of *Staph. aureus, coagulase negative staphylococci* and *Escherichia coli* intramammary infections (Arsenopoulos et al., 2018). These evidences indicate an increase in mastitis and fly populations during the summer months, but the relationship between the two is unknown in lactating dairy cows housed on pasture in the southeastern United States.

Subclinical and Clinical Mastitis

Mastitis, an inflammation of the mammary gland, can be categorized as either subclinical or clinical; with subclinical being defined as a cow having a somatic cell count (**SCC**) > 200,000 cell/mL and clinical defined as physical signs such as discoloration in the milk or swelling of the udder. Somatic cells are used as an indicator of an intramammary infection (**IMI**) because somatic cells consist of cells from the immune system, including neutrophils, lymphocytes, macrophages, eosinophils, and some epithelial cells (Kehrli and Shuster, 1994). Therefore, an increase in somatic cells are an indicator of the inflammatory response to an infection. There are many factors associated with the prevalence of mastitis, such as lactation, parity, management style, and season. Breen (2009), found that increased parity, decreased stage of lactation, cows with dirty udders, and udders with severe hyperkeratosis were both cow and quarter risk factors associated with the development of clinical mastitis (Breen et al., 2009). Hogan (1989), when monitoring low SCC herds, also determined that the mean rate of clinical mastitis cases was highest during the summer and decreased throughout the fall and winter to a low in spring ($P < 0.05$).

Parity can have a great influence on mastitis in dairy cattle. Older cows had a higher risk of mastitis than did younger cows (Gröhn et al., 1995). Periparturient heifers are less likely to have had to a previous case of clinical mastitis and older cows may have concurrent health problems increasing the risk of mastitis (Breen et al., 2009). Older cows may have been exposed to multiple cases of mastitis throughout their lifetime and have immunosuppression due to age, reducing the defense mechanisms in place to fight the infection.

The interaction of parity and stage of lactation can greatly affect SCC. An increase in SCC in later lactation has been found to be less for first parity cows than for later parity cows

(Wiggans and Shook, 1987). Hogan (1989), determined approximately 31% of total clinical cases occur in the 1st month of lactation, with 19.9% of clinical cases occurring within the first 7 days of lactation (Hogan et al., 1989). Clinical mastitis also developed after the first 30 d of lactation in cows that recorded a SCC > 199,000 cells/mL (Breen et al., 2009). Cows that are in the first month of lactation have increased odds to develop a first case of clinical mastitis in lactation before next monthly sample collection compared to those in lactation 6 months and above (Breen et al., 2009). In regard to SCC, late-lactation cows are more likely to develop or maintain a high SCC (Braund and Schultz, 1963, Olde Riekerink et al., 2007, Hagnestam-Nielsen et al., 2009). It was also determined that higher yielding cows had a higher risk of mastitis than did lower yielding cows (Gröhn et al., 1995). This could be due to the opening of the teat sphincter due to increased pressure in the udder causing an opening for organisms to enter.

Management style and housing also has an effect on mastitis. Barkema (1999), when observing management style and its association with BMSCC, found that farms with herds that had a low BMSCC had better hygienic conditions ($P < 0.001$). Farmers in the clean and accurate group were good record keepers, accurate in sampling for clinical mastitis, knew the cows better, and had better overall hygiene (Barkema et al., 1999). Rowbotham (2015) also determined that bulk milk somatic cell score (BMSCS) was lowest for farms using inorganic bedding, when new bedding was added to the stalls at intervals greater than one week, and teats were dried before attachment of the milking unit (Rowbotham and Ruegg, 2015). Differences in management style can describe the variation in prevalence of mastitis between farms.

Season also has a significant effect on the prevalence of mastitis and an increase in SCC in dairy herds (Bishop et al., 1980, Hogan et al., 1989, Olde Riekerink et al., 2007, do Amaral et

al., 2011, Shock et al., 2015). Hogan (1989), when monitoring low SCC herds, found that the mean rate of clinical mastitis cases was highest during the summer and decreased throughout the fall and winter to a low in spring. With summer comes an increase in heat and humidity that provide optimal environmental conditions for bacterial growth. Heat stress also causes immunosuppression in cows, increasing the risk of intramammary infections (do Amaral et al., 2011). When observing seasonality changes in BMSCC, individual cow SCC, and incidence rate of clinical mastitis, BMSCC was found to peak in August to September (Olde Riekerink et al., 2007, Rowbotham and Ruegg, 2015). Forty-eight to 71% of herds experience an increase in BMSCC during the summer (Shock et al., 2015). The probability of cows maintaining a high individual cow SCC was highest in August and May (Olde Riekerink et al., 2007). Bishop (1980), also found that cases of clinical mastitis were much higher in summer months, as compared spring and fall (Bishop et al., 1980).

Pathogen-specific Mastitis

The type of pathogen causing the infection also has an affect on the severity of the infection. *Staphylococcus* species is one of the most frequently isolated organisms on farm (Hogan et al., 1989, Levison et al., 2016). *Staphylococcus* species was the bacterial group most frequently isolated from quarters at calving (11.9%) and at dry off (13.5%) (Hogan et al., 1989). *Staph. aureus* is one of the most frequently isolated organisms on farm. *Staph. aureus* is frequently found in milk samples and heifer colostrum samples (Anderson et al., 2012). Sampimon (2009) determined the prevalence of coagulase negative staphylococci (**CNS**) IMI was estimated at 10.8% (95% CI: 8.5- 13.8%) at quarter level and 34.4% (95% CI: 31.5- 37.3%) at cow level, making it the most frequently isolated group of pathogens. Of the CNS species identified, the most frequently isolated species is *Staph. chromogenes* (Sampimon et al., 2009,

Supré et al., 2011). *Staph. chromogenes* causes persistent infections, but has a minimal effect on milk weight (Supré et al., 2011, Moroni et al., 2018). Lastly, *Staph. hyicus* and *Staph. agnetis* are isolated less frequently than *Staph. aureus* and *Staph. chromogenes* but are commonly observed. *Staph. hyicus* is a coagulase-variable staphylococcal species that is part of the commensal flora of various animals and is commonly isolated from bovine milk (Trinidad et al., 1990, Gillespie et al., 2009). Next to *Staph. chromogenes, Staph. hyicus* is a frequently isolated coagulase variable *Staphylococci.* When comparing mastitis pathogens between years, *Staph. hyicus* remained highest in the summer regardless of the year (Luc et al, 2019). *Staph. agnetis* is a gram positive, coagulase variable *Staphylococci*. *Staph. agnetis* can be misidentified as *Staph. hyicus* and other *Staph* organisms when using the *API Staph. System* as compared to PCR (Adkins et al., 2017).

Staph. aureus. *Staph. aureus* is one of the most common contagious types of *Staphylococci* that cause mastitis. *Staph. aureus* is a gram-positive contagious cocci that can be isolated from various areas of the farm such as housing, feed, other animals, water and insects (Roberson et al., 1994, Gillespie et al., 1999, Anderson et al., 2012). S*taph. aureus* causes diverse degrees of severity, ranging from severe mastitis with systemic signs to mild local infections. Clinical response to *Staph. aureus* intramammary infections is typically very mild and most often not clinically observable as the majority of intramammary infections are subclinical infections (Schukken et al., 2011). A cow infected with *Staph. aureus* mastitis can also have clinical fare-up episodes. The initial infection begins with severe clinical signs, increasing the cow's SCC and clotting in milk. Within a few days, the clinical signs may subside so the condition becomes subclinical with *Staph. aureus* still present in the udder (Rainard et al., 2018).

When observing the prevalence of mastitis pathogens, *Staph. aureus* is one of the most prevalent isolates cultured (Østerås et al., 2006). *Staph. aureus* has been predominantly found on heifer body sites, specifically on heifers > 12 months (Roberson et al., 1994). *Staph. aureus* is frequently found in milk samples and heifer colostrum samples (Anderson et al., 2012). The rate of *Staph. aureus* is found to be higher in right quarters, quarters that had recovered from *Staph. aureus* or *Strep. uberis* infections, quarters exposed to other *Staph. aureus* infected quarters in the same cow, and in quarters with extremely callused teat ends (Zadoks et al., 2001). Prevalence of *Staph. aureus* also decreases with an increase in days in milk (**DIM**; eg. days post calving) (Østerås et al., 2006). The highest prevalence of *Staph. aureus* can be observed from May to July (Østerås et al., 2006). *Staph aureus* is the most prevalent in the early stage of lactation in first lactation cows and lowest in second lactation cows (Østerås et al., 2006). Significant milk production loss was observed with cows infected with *Staph. aureus* and persisted until at least 70 days after diagnosis (Vanselow et al., 2006).

Staph. aureus contains surface proteins that promote adherence to damaged tissues, binding proteins that help avoid immune responses and promote iron uptake, and enzymes such as coagulase and collagenase (Oviedo-Boyso et al., 2007). *Staph. aureus* secretes a number of proteins that inhibit or delay the effects of the innate immune system (Rooijakkers et al., 2005). *Staph. aureus* also has numerous virulence factors that prevent the host immune system from acting effectively. These virulence factors include the production of antiphagocytic factors, such as protein A and a capsule, adhesion to mammary epithelial cells, intracellular survival in macrophages and mammary epithelial cells, and the production of endotoxins, bacterial superantigens and proteases (Dego et al., 2002).

Tumor necrosis factor-α (TNF- α) is known as a proinflammatory cytokine with both beneficial and injurious properties. Macrophages, lymphocytes, neutrophils and epithelial cells have been shown to produced TNF-α. TNF-α is undetectable in healthy quarters, but elevated concentrations of this cytokine have been detected in both milk and blood after infection. An increase in TNF- α has not been found in cows infected with *Staph. aureus* (Riollet et al., 2001).

The cytokine interferon-γ (IFN- γ) is critical for host immunity against intracellular pathogens. Lymphocytes, natural killer cells, and cells of monocytic lineages have been found to produce IFN- γ. INF-γ enhances the microbicidal activity of these immune cells by increasing receptor mediated phagocytosis, inducing respiratory burst activity, and priming nitric oxide production, while upregulating cell-surface MHC class I molecule expression (Schroder et al., 2004). Mammary glands infected with *Staph. aureus* have been found to have increases in IFN-γ mRNA (Riollet et al., 2001). In those mammary glands, IFN-γ was found to peak at 48 hr, with a concentration of 20 ng/mL (Riollet et al., 2001).

The recruitment of neutrophils, lymphocyte activation, and cytokine production characterizes an infection with *Staph. aureus* (Oviedo-Boyso et al., 2007). When the infection establishes itself, the recruitment of CD8+ lymphocytes predominates in the mammary gland (Götz, 2002). When bovine epithelial cells were stimulated with *Staph. aureus*, the TLR2 receptor was stimulated. Mammary epithelial cells that were challenged with *Staph. aureus* showed a significantly lower TLR4 mRNA level compared to those infected with *Eshcerichia coli*. Bovine epithelial cells have been found to be responsive to lipoteichoic acid, protein A and α-hemolysin that are found in association with *Staph. aureus*. The response of the epithelial cells to *Staph. aureus* is associated with AP-1 and interlekukin (IL)-17A signaling pathways (Gilbert et al., 2013). When a dairy cow becomes infected with *Staph. aureus*, tumor necrosis factor

(TNF)-α mRNA expression and IL-1β in mammary cells increases. A significant increase in transcription of TNF-α has been found in clinical cases, but then decreased 8 hours later. The decrease in TNF-α coincided with an increase in IL-1β production (Bannerman et al., 2004). These results suggest both TNF-α and IL-1β play an important role in the early stages of mastitis. An increase in the cytokines interferon-γ (IFN- γ), IL-1_{β}, IL6, IL12, transforming growth factor-α (TGF-α) have been detected in mammary epithelial cells challenged with *Staph. aureus* (Riollet et al., 2001, Bannerman et al., 2004). The cytokines IL8 and IL10 on the other hand, have not been detected with a *Staph. aureus* infection (Riollet et al., 2001, Bannerman et al., 2004). IL-8 is important in that it recruits neutrophils to the site of infection and also stimulates phagocytosis. The expression of various cytokines and immune factors when the mammary gland is infected with *Staph. aureus* can determine how severe and persistent the infection is.

There are various interleukin cytokines associated with *Staph. aureus* infections. IL-1 is a proinflammatory cytokine that induces fever (Dinarello, 1998). Sources of IL-1 include cells such as monocytes, macrophages, dendritic cells, lymphocytes, endothelial cells, and fibroblasts. IL-1 can be expressed as either IL-1_{α} or IL-1_{β}. IL-1_{α} is generally found intracellularly, whereas IL-1_{β} is generally secreted. Cows chronically infected with *Staph. aureus* have been found to have an increase in IL-1_{β} transcription (Riollet et al., 2001). IL-6 is expressed by lymphocytes, monocytes, macrophages, neutrophils, endothelial cells, epithelial cells and fibroblasts. IL-6 can also induce fever, while differentiating B-cells, activating T-cells, and enhancing proinflammatory responses of neutrophils. IL-6 mRNA transcription has also been found to increase in cells isolated from cows infected with *Staph. aureus* mastitis (Riollet et al., 2001). IL-8 is a chemotactic cytokine that is also upregulated in response to infection. Endothelial and epithelial cells, fibroblasts, neutrophils, and T-cells are all able to produce IL-8. Gram-positive

bacterial infections have been shown to induce a delayed or absent IL-8 response. When various strains of *Staph. aureus* were used to experimentally infect cows, increases in milk IL-8 were unable to be detected (Riollet et al., 2001). IL-10 plays an important role in limiting inflammation by inhibiting the production of proinflammatory cytokines, chemokines, and eicosanoids. IL-10 is produced by type 2 helper T cells (TH2), B cells, eosinophils, and mast cells. An increase in IL-10 was not observed in response to *Staph. aureus* intramammary infections (Bannerman et al., 2004). The lack of IL-10 response can also correspond with the lack of induction of TNF-$_{\alpha}$. Cows with the most persistent infections had a delayed induction of IL-10 (Riollet et al., 2001). Lastly, IL-12 is a cytokine that is produced by monocytes and dendritic cells. IL-12 enhances the cytotoxic activity of cytotoxic T cells and natural killer cells. IL-12 is also very important in altering the balance between TH1 and TH2 responses by promoting the differentiation of T cells into IFN-γ producing TH1 cells. An increase in mRNA abundance has been detected in cells isolated from cows with *Staph. aureus* mastitis (Riollet et al., 2001).

Transforming Growth Factor-α (TGF-α) has been found to mediate wound healing, epithelial growth, angiogenesis, and mammary gland morphogenesis. Increased concentration of TGF-α has been detected after intramammary infection by a variety of bacterial pathogens (Kauf et al., 2007). TGF-α concentrations are typically sustained for a longer period of time after infections compared to other cytokines. In contrast, transforming growth factor-β (TGF- β) is known for its assistance with cell growth and differentiation. TGF-β1 and TGF-β2 are both expressed in bovine milk. Increases in both TGF-β1 and TGF-β2 have been detected when the mammary gland was infected with *Staph. aureus* (Bannerman et al., 2004). Increases in both TGF-β1 and TGF-β2, though, were not detected until >32 h after initial infection.

Coagulase Negative Staphylococci. Coagulase negative *Staphylococci* (CNS) are one the most prevalent organisms isolated from cow's milk. CNS are part of the normal flora of the skin of the teat and external orifice of the streak canal (Moroni et al., 2018). The prevalence of CNS intramammary infection has been estimated at 10.8% (95% CI: 8.5- 13.8%) at quarter level and 34.4% (95% CI: 31.5- 37.3%) at cow level, making it the most frequently isolated group of pathogens (Sampimon et al., 2009). The majority of new infections at calving are caused by CNS (64.3%) (Supré et al., 2011). The prevalence of CNS intramammary infection has been found to be higher in heifers compared to older cows (Sampimon et al., 2009). The highest prevalence of CNS species has been observed from April to July (Østerås et al., 2006). The bacteria most commonly causes subclinical mastitis, with minimal signs of infection and a typically fast cure rate. CNS causes a slight increase in SCC, but has minimal effect on milk yield (Moroni et al., 2018). The most common species of CNS include *Staph. chromogenes, Staph. hyicus,* and *Staph. epidermidis*. When cows were challenged with either *Staph. simulans* or *Staph. epidermidis*, cows' temperature peaked just 6 h after infection, swelling of the udder decreased after 36 h and SCC increased until 27 h post challenge (von Eiff et al., 2002). When cows are infected in mid-lactation, SCC was seen to double in amount compared to cows that were infected in late lactation (Leitner et al., 2012).

Of the CNS species identified, the most frequently isolated species is commonly *Staph. chromogenes* (Sampimon et al., 2009, Supré et al., 2011). *Staph. chromogenes* is commonly isolated from bovine milk and colonizes on teat end surfaces; with 10% of heifer teat apices colonized with *Staph. chromogenes* (De Vliegher et al., 2003). Quarters infected with *Staph. chromogenes* had a higher SCC ($P < 0.05$) than culture-negative quarters, averaging 192,000 cells/mL (Sampimon et al., 2009). *Staph. chromogenes* is also known to cause persistent

infections (Supré et al., 2011). On average, *Staph. chromogenes* infections last approximately 147.8 (89- 303) d (Supré et al., 2011).

Staph. hyicus is a gram-positive coagulase-variable staphylococcal species that is part of the commensal flora of various animals and is commonly isolated from bovine milk (Trinidad et al., 1990, Gillespie et al., 2009). When determining the prevalence of coagulase positive staphylococci, 17.7% of the samples were *Staph. hyicus* (Roberson et al., 1996). *Staph. hyicus* is capable of inducing chronic, low-grade intramammary infections. When differentiating *Staph. hyicus* using the API Staph method, it can commonly be misidentified. When the *tuf* gene was used to differentiate bacteria species, 42 coagulase positive isolates that were previously identified as *Staph. hyicus* were identified as *Staph. agnetis* (Adkins et al., 2017). *Staph. agnetis* is a gram positive, coagulase variable *Staphylococci*. *Staph. hyicus* and *Staph. chromogenes* 16S rRNA gene sequences have been found to exhibit 99.7% and 99.1% identity to *Staph agnetis*, respectively (Taponen et al., 2012). The overall prevalence of *Staph. agentis* is low, ranging from 0.0% to 2.2% (Adkins et al., 2017).

Mastitis on Organic Dairy Herds

Organic dairy products have seen an increase in demand and in result, organic certification is becoming highly regulated. The organic system places an emphasis on reducing stress of the animals. A comprehensive list of organic dairy regulations can be found on the USDA website (USDA, 2020). In short, dairy products must come from animals that have been managed in the organic system for at least one year. While under organic production, all animals must be receiving 100% organic feed (USDA, 2020). Housing in these organic systems must provide access to the outdoors year-round and total confinement of an animal older than 6 months of age is prohibited (USDA, 2020). The animal should receive at least 30% of its dry

matter intake (DMI) from pasture during the grazing season, which should not be shorter than 120 days (USDA, 2020). In the United States, national standards for organic production includes the usage of antimicrobials to treat dairy cattle results in permanent loss of organic status of the animal (USDA, 2013). There are currently minimal products approved by the US Food and Drug Administration that can be used for treatment of mastitis on organic dairy farms.

In the United States, organic farmers treat clinical mastitis using a variety of alternative therapies including whey-based products, botanicals, vitamin supplements, and homeopathy (Ruegg, 2009). Other commonly used products include garlic tincture, aloe vera, and vitamin C (Pol and Ruegg, 2007). Organic farms have been found to have little to no difference in BMSCC compared to conventional herds (Hardeng and Edge, 2001, Zwald et al., 2004, Cicconi-Hogan et al., 2013). It can also be observed that subclinical mastitis between organic (20.8%) and conventional (23.3%) farms in North Carolina did not differ significantly (Mullen et al., 2013). Mean somatic cell score (SCS), also, did not differ between organic (3.3 ± 0.2) and conventional (3.5 ± 0.2) herds (Mullen et al., 2013). Levison reported that the incidence rate of clinical mastitis was higher on conventional farms (23.7 cases per 100 cow-years) when compared to organic farms (13.2 cases per 100 cow-years) (Levison et al., 2016). The presence of *Staph. aureus* in the bulk tank has been found to be higher on organic farms compared to conventional farms (Sato et al., 2004, Pol and Ruegg, 2007, Cicconi-Hogan et al., 2013).

The mean SCC of bulk-tank milk samples were 94 x 10^3 cells/ mL (SD: 111 x 10^3 cells/ml) in the summer and 79 x 10^3 cells/mL (SD: 86 x 10^3 cells/ mL) in the winter (Busato et al., 2000). When comparing SCC between cows housed on pasture and cows that were confined to a barn, the mean SCC was 142 x 10^3 cells/mL (SD: 210 x 10^3) for cows on pastures; whereas SCC of cows staying in home barns was 87 x 10^3 cells/mL (SD: 73 x 10^3 cells/mL). Cows that

were sampled when staying on pasture had significantly higher odds having subclinical mastitis than cows staying in home barns (Busato et al., 2000).

Milk Yield and Stress

When mastitis occurs in early lactation, milk production is affected for an extended period and results, on average, in a 911 kg reduction over the entire lactation. When mastitis occurs in mid- to late lactation, the average loss is 850 kg over the entire lactation (Lescourret and Coulon, 1994). The production at mastitis onset is a determining factor on the amount and pattern of milk production loss induced (Lescourret and Coulon, 1994). The natural log of SCC is negatively correlated with milk yield. A cow with relatively high SCC (250,000 cells/mL) compared to a cow with a relatively low SCC (50,000 cells/mL) produces on average, 1.6 kg/d less milk (Potter et al., 2018).

When investigating pathogen-specific impacts of mastitis on milk production, cows with a *Staph. aureus* intramammary infection were found to lose 2.3 kg/d milk yield (Heikkilä et al., 2018). When *Staph. aureus* mastitis was diagnosed between 54 and 120 d in milk, milk loss per day was found to be 1.4 kg. Cows with clinical and subclinical *Staph. aureus* mastitis lose 2.3 and 2.2 kg milk yield per day, respectively. Cows infected with CNS are also expected to lose 1.8 kg of milk per day; whereas no significant decreases in milk yield were observed with cows with subclinical CNS (Heikkilä et al., 2018). CNS species has been found to have minimal effects on milk yield (Moroni et al., 2018).

Daily temperature-humidity index (THI) is negatively correlated to milk yield ($r = -0.76$, $P < 0.01$). When THI increases from 68 to 72, milk production decreases by 21% (Bouraoui et al., 2002). When comparing breed differences, Holstein cow milk yield decreased from 34.8 kg/d to 30.4 kg/d when exposed to severe heat stress ($THI \geq 90$) ($P < 0.0001$); whereas Jerseys cows

decreased their milk weight from 25.9 kg/d to 23.8 kg/d ($P < 0.0001$) (Smith et al., 2013). The THI values one ($r = -0.83$), two ($r = -0.87$), and three ($r = -0.89$) days prior had a greater effect on milk yield than the same day measure (Bouraoui et al., 2002).

Horn Flies and Mastitis

There are various types of flies found on farm and on the animal. The most common fly types include the stable fly, house fly, horn fly, and face fly. These flies are considered filth flies which means breeding occurs in a wide variety of moist, fermenting organic matter (Christensen, 1982). The stable fly (*Stomoxys calcitrans*) is commonly found on the legs of pastured cattle and feed with their head facing up toward the dorsal midline. Although closely resembling the common house fly, stable flies have piercing mouth-parts that project from the front of the head which delivers a painful bite (Christensen, 1982). In contrast to the stable fly, the house fly (*Musca domestica*) has sponge-like mouthparts that are used to feed on a variety of foods and then dissolve solid foods with regurgitated liquids. Similarly, face flies (*Musca autumnalis*) also have spongy mouthparts but are found feeding on mucous secretions from the eyes and noses of cattle. Face flies have been found to assist in the transmission of *Moraxella bovis,* the causative agent of pinkeye.

Lastly, and quite possibly the most important fly found on cattle, are horn flies (*Haematobia irritans*). Horn fly numbers have been found to be highest in permanent, low-lying, moderately to extensively sheltered pastures (Jensen et al., 1993). An increase in horn fly populations are seen during the summer and are highly correlated with relative humidity ($r = 0.53$), rainfall ($r = 0.67$), and average temperature ($r = 0.21$) (Lima et al., 2003, Maldonado-Siman et al., 2009). According to Morgan (1964), the ideal environment for survival of a horn fly is temperature between 23 to 27 °C, relative humidity of 65 to 90%, scattered light rain showers,

and no wind (Morgan, 1964). Tennessee and Kentucky create the ideal conditions for the horn flies during the summer months.

Horn flies are typically found on the backs and shoulders of pastured cattle. They remain on the cattle for the majority of their lifetime, with females only leaving to deposit eggs in fresh manure piles (Bruce, 1940). The eggs hatch in 16 to 20 hours and, within 4 to 5 days, adults are feeding on the animal (Bruce, 1940). The entire lifecycle of the horn fly is approximately 12 to 14 days. Horn flies are obligatory blood feeders and when feeding, face the ground and orient the wings at a 45-degree angle. Horn flies are most active during the spring and summer and overwinter as pupae beneath dung pats (Lysyk, 1999). During extremely hot weather, cold weather, and rainy periods, horn flies will move to the underside of the animal, near the udder (Morgan, 1964). This becomes a concern because horn flies are known to carry mastitis-causing pathogens.

Horn flies are known to carry *Staph. aureus*, which is a major mastitis causing pathogen. Horn fly samples have been found to frequently test positive for *Staph. aureus*, with over one half, or 55.8% of the samples testing positive (Anderson et al., 2012). Rumen, environmental, symbiotic, and pathogenic bacteria have been found to be associated with the horn fly using the bacterial 16S tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) method. Among the 37 OTUs (Operational Taxonomic Unit) identified in horn fly males, *Wolbachia* was the most abundant (52.4%) bacterial species in the adult horn fly. Among the 25 OTUs identified in females, *Staph. aureus* was the most abundant bacteria in the adult female horn fly (Palavesam et al., 2012). Horn flies have been found to cause *Staph. aureus* mastitis, specifically in heifers (Gillespie et al., 1999). *Staph. aureus* titers have been found to increase 2- to 3- fold during the period when fly populations increase drastically and teat skin conditions worsen. The increase in

titers began in April and continued through September, corresponding with the increase in fly populations (Ryman et al., 2013). Anderson (2012) indicated that it was 5 and 8 times more likely to find *Staph. aureus* in horn flies compared to colostrum and milk samples, respectively (OR = 5, 95% CI= 1.69 – 6.78; OR= 8, 95% CI= 4.32- 14.93) (Anderson et al., 2012). Identical genotypes were also obtained from horn flies, heifer colostrum samples, and cow milk samples (Gillespie et al., 1999, Anderson et al., 2012). With the common subtype found between heifers and horn flies, it was suggested that horn flies play a major role in transmission of *Staph. aureus* to heifers. When horn flies were exposed to bacteria and then exposed to heifers, summer mastitis developed in those heifers. The bacteria species found in these heifers corresponded to the bacteria fed to the horn flies (Chirico et al., 1997).

An increase in horn fly numbers could cause a reduction in milk weight. Mays et al. (2014) found a negative relationship between horn fly numbers and milk yield in beef cattle. Milk yield has also been found to decrease ($P < 0.05$) by 0.72, 0.68, and 0.71 kg/d per unit increase in log horn fly count in May, June, and July, respectively (Mays et al., 2014). In contrast, Wolley et al (2018), did not find a relationship between milk weight and horn fly numbers when cows were treated with essential oil fly repellent to reduce horn fly numbers (Woolley et al., 2018).

Horn flies are also known to transmit the filarial nematode *Stephanofilaria stilesi*. Horn flies pick up the nematode larvae when feeding on stephanofilarial lesions (Hibler, 1966). *Stephanofilaria stilesi* causes stephanofilariasis in cattle, which is circumscribed dermatitis along the midventral line of the body (Hibler, 1966). Cattle that are 3 to 5 years of age are most likely to have stephanofilariasis, with prevalence decreasing by 5 to 7 years old (Hibler, 1966).

Although no detrimental effects are associated with stephanofilariasis, reducing horn fly numbers on farm can lower the prevalence of stephanofilariasis on farm.

The distribution of the number of flies within a herd can depend on the number of fly resistant or fly-susceptible heifers. Possible factors that make fly-resistant heifers less fly attractive include color, temperature, thickness of hide, hair density or amount of sebum in the hair coat, or the ability of the individual fly to land successfully (Jensen et al., 1993). Steelman et. al (1997) determined that an increase of 100 hairs per cm^2 was associated with the reduction of horn flies in pastured beef cattle (Steelman et al., 1997). It was also determined that each increase of 0.25 g of sebum per 929 cm^2 resulted in a decrease of 9.2 horn flies per steer (Steelman et al., 1997). Another possibility is that fly numbers are determined by dynamic differences in chemical factors emitted by cattle (Jensen et al., 2004).

Control Methods for Flies

As previously stated, horn flies are known to carry numerous pathogens that can greatly affect the production of dairy cattle. Implementing some type of control strategy to reduce horn fly numbers on farm is important to increase overall production on farm. Heifers from herds using fly control had lower prevalence of intramammary infections than herds without fly control (Nickerson et al., 1995). When looking at the prevalence (%) of mastitis in 600 unbred and primigravid heifers in 5 herds with (n= 3) and without (n= 2) fly control, 55.2% of the heifers without fly control had *Staph. aureus* mastitis, compared to 5.6% of heifers that had fly control (Nickerson et al., 1995). The product available for on-farm fly control varies between conventional and organic systems, with organic systems having a stricter set of regulations on what products can be used.

The use of deltamethrin in commercial herds reduced fly populations and ultimately facilitated a decrease in *Staph. aureus*, CNS, and *E. coli* infections while also decreasing SCC (Arsenopoulos, 2018). Deltamethrin application reduced the fly burden within the same day by 88%. The isolation rate of *Staph. aureus* in milk samples of cows treated with deltamethrin was decreased from day 0 to 30. SCC of cows treated with deltamethrin, followed a stable reduction rate from Day 0 to 30, respectively (1029 (± 867.11)*10^3 cells/mL; 546.82 (± 123.23)*10^3 cells/mL) (Arsenopoulos et al., 2018). This shows that if effective fly control is implements on farm, milk quality could improve. Pour-on formulation and diazinon-impregnated ear tags are also a common control strategy for horn flies on farm. Cattle with an ear tag averaged < 5 flies per animal, up to 55 d after treatment. Flies were reduced 94% (± 1.74) during the first 52 d following application of the ear tag (Lysyk and Colwell, 1996). Cattle that are treated with pour-on ivermectin had significant reduction in horn fly numbers ($P < 0.05$) for approximately 6 weeks (Marley et al., 1993).

Limited methods are available for fly control on organic dairy herds. One strategy for fly control on an organic system is the use of a walk-through fly vacuum. As cows walk through the vacuum, flies are brushed off the face, flank, and back with hanging flaps and blown off the belly, udder, and legs from one side. Those removed flies are vacuumed from the air into a chamber from vacuum inlets opposite the blower and above the cows. When the trap was placed on farm, it removed 410,000 horn flies from cattle within the first week and an additional 457,000 horn flies the next week (Denning et al., 2014). The trap reduced mean horn fly densities from 775 to 150 flies per cow by the third week of use. In a similar study, when a CowVac was placed on farm, horn fly numbers on the cows were lowered by 44% ($P < 0.05$) compared with the absence of a trap (11.4 vs 20.5 flies/cow-side) (Kienitz et al., 2018). These

results indicate the trap was effective in reducing horn fly numbers on cows and reduced horn fly population rates during the pasture season in organic dairy production systems. The traps captured a greater ($P < 0.05$) number of horn flies (71.9 ± 14.4 vs 15.1 ± 16.7 flies/ cow per day) for farms that had no housing compared with farms that had access to housing, respectively (Kienitz et al., 2018). Essential oils are also used for fly control and include oils from basil, geranium, balsam fir, lavender, lemongrass, peppermint, pine and tea tree. Essential oils can repelled > 75% of the flies on the treated area up to 6 hours on pastured cattle (LaChance and Grange, 2014).

Conclusion

Knowing mastitis is the most common disease found on US dairy farms in the and it is also the number one use for antibiotics on commercial dairy farms, it is critical to understand the risk factors influencing mastitis on farm. Fly populations increase during the summer, which could partly contribute to the increase in mastitis during that time. The relationship between the two is unknown in lactating dairy cows housed on pasture in the southeastern United States where extended periods of heat and humidity provide optimal growth conditions for horn flies and needs to be investigated further.

CHAPTER II

Horn Fly Effects on Milk Quality and Yield in Organic Dairy Herds

Abstract

Summer mastitis continues to be a challenge on many dairy farms and this may be linked to increased horn fly populations during summer months, but detailed knowledge regarding the relationship between the two is limited. The objective of this study was to determine the association between horn fly numbers, somatic cell count (SCC), and milk yield on organic dairy herds. We hypothesized that as horn fly populations increase SCC will also increase, while milk yield will decrease. Four USDA-certified organic herds located in Tennessee and Kentucky were enrolled in the study, with an average herd size of 55 lactating cows. Sampling began in May 2019 and continued through October 2019. Dairy Herd Information Association (DHIA) tests were used to collect an individual cow's SCC and milk weight (kg). Testing occurred every 28 days on the entire lactating herd on each farm, and samples were processed in the DHIA lab. Fifteen focal cows from each herd (n = 69) were monitored for individual horn fly numbers. A Nikon Coolpix P1000 camera was used to take digital image of horn flies on an individual cow's dorsal and ventral midline every other week. Images were then processed with a convolutional neural network trained to segment the salient cow in each image. A separate network then was used to locate flies and provide a total fly count for the given area. The total number of flies from each area were then added together to get a total number of flies per cow on a given date. To test if logSCC or milk weight were affected by fly numbers on cows, multivariable regression with generalized linear mixed models were used with fixed effects of total flies per animal on a given date, temperature humidity index (THI), season, parity, days in milk (DIM), and random effects of cow(farm), and a repeated measure of date. Unexpectedly, no relationship was observed between horn fly numbers and log SCC. DIM had the most significant effect on the change in SCC throughout the summer. A significant negative relationship was found between horn fly

numbers and milk weight. As horn fly populations increased, milk weight decreased. With this information, the reduction in milk weight during the summer is at least partly due to an increased number of horn flies. These data show the importance of fly control on the farm during the summer to improve milk yield.

Introduction

Summer negatively affects both milk quality and yield on dairy farms. Hogan and colleagues (1989) observed clinical mastitis was highest during the summer months and decreased throughout the fall and winter months (Hogan et al., 1989). When observing seasonality changes in bulk milk somatic cell count (BMSCC), individual cow SCC and incidence rate of clinical mastitis, all measures were found to increase during the summer, with BMSCC count peaking in August to September (Olde Riekerink et al., 2007, Rowbotham and Ruegg, 2015). Organic farms have little to no difference in BMSCC compared to conventional herds (Hardeng and Edge, 2001, Zwald et al., 2004, Cicconi-Hogan et al., 2013). Our research also indicates cows on organic dairies have a significantly greater probability of experiencing subclinical mastitis (SCC > 200,000 cells/ml) in the summer relative to spring, fall, or winter (Couture et al., 2018). An increase in daily THI that occurs in summer is also negatively correlated to milk yield ($r = -0.76$). When THI increases from 68 to 72, milk production decreases by 21% (Bouraoui et al., 2002). Horn fly numbers increase in the summer (Mays et al., 2014); therefore, there may be a negative relationship between horn fly number and milk weight. Mays et al (2014) determined milk yield can decrease with a horn fly infestation in beef cows depending on sire breed and month of lactation (Mays et al., 2014). Milk yield decreased approximately 0.7 kg/d per unit increase in log horn fly count in May, June, and July (Mays et al., 2014).

Active during the spring and summer months, horn flies (*Haematobia irritans*) are obligatory ectoparasites found blood feeding on the backs and shoulders of pastured cattle where they remain for the majority of their adult lifetime (Bruce, 1940, Lysyk, 1999). During extremely hot weather, cold weather, and rainy periods, horn flies will move to the underside of the animal,

near the udder (Morgan, 1964). This becomes a concern because horn flies are known to carry mastitis-causing pathogens, such as *Staph. aureus*. Horn flies have been found to cause *Staph. aureus* mastitis, specifically in heifers (Gillespie et al., 1999) and were frequently collected from the field and tested positive for *Staph. aureus* (Anderson et al., 2012). Anderson (2012) indicated that horn flies were 5 and 8 times more likely to be *Staph. aureus* positive than colostrum and milk samples, respectively (Anderson et al., 2012). Knowing horn flies feed on the udders and teats of lactating dairy cattle, it was noted that *Staph. aureus* antibody titers increased 2- to 3-fold from April through September when fly populations increase drastically and teat skin conditions worsen (Ryman et al., 2013). Identical *Staph. aureus* genotypes were also obtained from horn flies, heifer colostrum samples, and cow milk samples (Gillespie et al., 1999, Anderson et al., 2012). With the common subtype found between heifers and horn flies, it was suggested that horn flies play a major role in transmission of *Staph. aureus* to heifers. When horn flies were exposed to bacteria and then exposed to heifers, summer mastitis developed in those heifers and the bacteria species found in the same heifers corresponded to the bacteria fed to the horn flies (Chirico et al., 1997).

Not only can horn flies transmit *Staph. aureus* to cattle, but an increase in horn fly numbers could cause a reduction in milk weight. Mays et al. (2014) reported a negative relationship between horn fly numbers and milk yield in beef cattle. Milk yield has also been found to decrease significantly by 0.72, 0.68, and 0.71 kg/d per unit increase in log horn fly count in May, June, and July, respectively (Mays et al., 2014). In contrast, a relationship between milk weight and horn fly numbers was not observed when cows were treated with essential oil fly repellent to reduce horn fly numbers (Woolley et al., 2018). Deltamethrin applications

reduced horn fly populations and burden; ultimately, facilitating a decrease in *Staph. aureus*, CNS, and *E. coli* infections while also decreasing SCC (Arsenopoulos et al., 2018).

In the southeastern United States, dairy cattle are exposed to a number of environmental problems such as mastitis, summer heat stress, and blood feeding by horn flies. It is known that summer heat stress effects lactating dairy cows housed on pasture by increasing SCC, summer incidence of mastitis, and decreased milk yield. The relationship between horn flies and SCC and milk yield is unknown in lactating dairy cows housed on pasture. Our hypothesis is that cows with greater horn fly numbers will have an increase in SCC and a decrease in milk yield.

Materials and Methods

The University of Tennessee Institutional Animal Care and Use Committee approved all animal- related procedures for this study (Protocol Number 2391). The study was conducted on four USDA certified organic herds, with the requirement that all farms participate in regular Dairy Herd Improvement (**DHI**) testing (TN Dairy Herd Improvement Association, Knoxville, TN; Mid-South Dairy Records, Springfield, MO). These four farms are located in Tennessee and Kentucky and were recruited through the University of Tennessee and University of Kentucky Extension Cooperatives. Sampling occurred during the months of May through October 2019.

Animals and Management

The average herd size between the four farms was 55 (SD ± 17) lactating cows. Individual herds were primarily Holstein, Jersey, or cross-breeds. Cows on all farms were milked twice daily. Morning milking occurred between 0500 and 0700h and evening milking occurred between 1700 and 1900h. Peppermint-based udder cream was used as needed to minimize the effects of clinical mastitis and swelling associated with freshening on all farms. No other treatments were used to treat clinical mastitis on any farm.

Tiestalls, compost bedded packs, or concrete bedded pens were the main source of housing when cows were not on pasture. Farm 1 housed cows in concrete pens, whereas Farms 2 and 3 housed cows in compost bedded packs. Farm 4 housed cows in a tunnel ventilated tiestall barn during the heat of the day. All herds had access to pasture and relied on pasture for more than 30% of their dry matter intake, as required by USDA organic certification. Grazing occurred at the minimum level or greater through the months of April through October, with the requirement that grazing must be ≥ 120 d. Silage, haylage, and concentrated feed was provided as supplementation to pasture and was delivered either directly before or after milking.

As for fly control measures, Farm 1 used a CowVac (Spalding Laboratories, Reno, NY) twice a day at the time of milking. Farm 2 used fly predators released twice a month starting in April through the end of October and applied essential oils designed for fly control to the cows twice a week. Farm 3 applied essential oils to the cows once a week as the only fly control method. Farm 4 used fly tape that ran the length of the tiestall barn and applied essential oils to the cows every 3 days.

Data Collection

THI Data. Weather data, which included temperature and humidity for each sample date, was accessed through an online database (Weather Underground, www.wunderground.com). The following equation was used to calculate THI from the temperature and humidity data collected (Ravagnolo et al., 2000); where T= air temperature (°C) and RH = relative humidity (%):

$$\mathbf{THI = (1.8T + 32) – [(0.55 - 0.0055RH) \times (1.8T - 26)]}$$

Sample dates were further categorized into season according to the astronomical definition, with spring beginning on March 21st, summer beginning on June 21st, and fall beginning on September 21st. One sample date for each farm occurred in spring, 3 samples dates

occurred in summer, and one sample date occurred in fall; the lone exception is farm 4, which left the study at the end of August.

Milk Data. Dairy Herd Information tests were used to collect an individual cow's SCC and milk weight (kg). Testing occurred on every 27 (± 5) days on the entire lactating herd on each farm with 6 collection dates for most participating farms. Farm 4 dropped from the study at the end of August, resulting in only 4 DHI collection dates. Milk samples from Farms 2, 3, and 4 were brought to the TN DHIA lab (Knoxville, TN) to be analyzed for SCC and fat and protein content. Samples from Farm 1 were collected and analyzed through Mid-South Dairy Records and results were provided directly to us.

Capturing Individual Animal Fly Counts. Fifteen cows from each herd (n = 69) were chosen to determine individual fly numbers. Cows were chosen based on stage of lactation and the potential to remain in the study throughout the sampling period. The mean (± SD) for DIM was 187 (± 65), whereas parity was 4 (± 2). If cows were dried off or culled, replacement cows were added to the group on the subsequent visit to keep the focal number at 15 total cows per visit. A camera, Nikon Coolpix P1000 16.0- Megapixel Digital Camera (Nikon, Tokyo, Japan), was used to take pictures of an individual cow's dorsal and ventral midline, resulting in two pictures per focal cow each visit. These pictures were processed with a convolutional neural network trained to segment the salient cow in each image. A separate network then was used to locate flies and provide fly counts on the dorsal and ventral midline. The total number of flies reported for the dorsal and ventral midline then were added together to get a total number of horn flies per focal cow on a given date. Methods and validation of this assay is presented in Psota and others (Psota, unpublished). Pictures were taken of individual cows every 12.8 d (± 5) on each farm, for a total of 12 visits; except for Farm 4 who had 8 visits total.

Statistical Analysis

To eliminate confounding issues, cows (n = 16) beginning the study with a SCC > 200,000 cells/mL were removed from the analysis at the beginning of the study because a SCC >200,000 cells/mL indicates a cow may have a subclinical infection present and inadequately represents the horn fly's association with an increased SCC. When SCC recovered and was ≤ 200,000 cells/mL, cows (n = 6) were added back into the analysis and remained there for the rest of the study. SCC was log transformed using $\log_{10}$ for analysis. Non-transformed values are reported in figures and tables to provide a more familiar format. In order to determine the average SCC, milk yield, and horn fly numbers for each farm, PROC FREQ within SAS 9.4 (Cary, NC) was used. In order to determine the correlation milk weight and horn fly numbers with sample date, PROC CORR was used (SAS 9.4).

To test variables related to logSCC and milk weight for an individual cow, multivariable generalized linear mixed models (PROC GLIMMIX) were developed using manual backward model selection. Fixed effects included total number of flies per cow on a given date, THI, season, parity, and DIM, with logSCC and milk weight included depending on the response variable used. Random effects included cow(farm) and the repeated measure of date. To determine herd level effects on logSCC and milk weight, multivariable regression with generalized linear mixed models (PROC GLIMMIX) within SAS 9.4 (Cary, NC) were developed using manual backward model selection. The fixed effects of the average number of flies on each herd, season, average DIM, and average parity were used. Average logSCC and average milk weight were also included depending on the response variable used. Random effect included farm and the repeated measure of date. Backward elimination was used to develop the most parsimonious informative model for each analysis. Significance was considered at $P \leq 0.05$.

Results

The mean SCC for the 15 focal cows on each farm throughout the study was 161,135 cells/mL (± 473,926 cells/mL); compared to a mean SCC of 241,064 cells/mL (± 524,510 cells/mL) for all lactating cows on each farm. Mean monthly herd and focal cow SCC varied by farm, with peaks seen in July and August (Table 2.1). Farm 1 had the highest herd average SCC at 316,874 cells/mL (± 728,250 cells/mL); whereas farm 3 had the lowest herd average SCC of 125,938 cells/mL (± 151,641 cells/mL). The mean milk weight was 13.0 kg (± 6.8 kg) throughout the study for the 15 focal cows on each farm. Farm 1 had the highest herd milk production of 20.4 kg (± 4.5 kg), whereas Farm 3 produced the lowest average of 9.1 kg (± 4.9 kg) of milk throughout the study (Table 2.2). Mean milk yield was negatively correlated with date of sample collection (r = -0.39; $P < 0.0001$).

The mean number of flies per cow throughout the study was 96.9 (± 94.7). Cows averaged 53.1 (± 69.4) flies on the dorsal midline and 52.1 (± 40.9) flies on the ventral midline. Mean number of horn flies was positively correlated with sample date, meaning horn flies continuously increased throughout the summer (r = 0.32; $P < 0.0001$) (Figure 2.1). Farm 3 had the greatest fly numbers, averaging 152.8 (± 139.1) flies per cow; whereas Farm 4 had the least number of flies per cow, averaging 46.3 (± 33.2) flies.

LogSCC and Horn Fly Numbers

The final model, when testing the effects on logSCC at the cow level, included DIM, parity, season, and milk weight (Table 2.4). DIM had the greatest effect on logSCC ($P = 0.003$). As DIM increased, logSCC increased as well (Figure 2.2):

logSCC= 4.52 + 0.003 (DIM)

Milk weight was also associated with the change in logSCC ($P = 0.006$). For every one unit increase in milk yield, logSCC decreased by -0.02 (se = 0.007). No relationship was observed between total number of horn flies per cow and logSCC ($P = 0.95$; Figure 2.3). When the model was run by farm, similar results were found, in that horn flies present on focal cows were not associated with the same cows logSCC. For Farm 1, parity ($P = 0.05$) was the only significant factor associated with logSCC. Season ($P = 0.04$) was the only significant factor associated with logSCC on Farm 3 and DIM ($P = 0.01$) was the only significant factor for Farm 4. No significant factors were associated with logSCC on Farm 2.

The only effects that remained in the model when testing the effects on logSCC at the herd level included average total number of horn flies per cow, season, and average milk weight (Table 2.5). There was no significant association with any fixed effects that best explained the variation.

Milk Weight and Horn Fly Numbers

We assessed a combination of factors for their relative importance in explaining changes in milk weight per cow. The effects that remained in the model for milk weight at the cow level included total number of horn flies per cow, DIM, parity, season, and logSCC (Table 2.4). A significant negative effect of mean horn fly numbers on milk weight was observed ($P = 0.003$). As horn fly numbers increased, milk yield decreased (Figure 2.4):

Milk Weight = 28.0 - 0.01 (mean horn fly number)

LogSCC ($P = 0.008$), DIM ($P = 0.02$), and season ($P = 0.04$) also were found to have a significant relationship with milk weight. When the model was run by individual farm, horn flies ($P = 0.003$; $P = 0.02$, respectively) were significantly associated with milk weight on farms 2 and

3. Season ($P = 0.01$) was the only significant factor associated with Farm 1 and season ($P = 0.004$), DIM ($P = 0.03$), and THI ($P = 0.05$) were the factors associated with milk weight on farm 4.

The effects that remained in the model for milk weight at the herd level included average number of horn flies per cow, DIM, and logSCC (Table 2.5). The tests revealed that logSCC ($P = 0.05$) and average number of horn flies per farm on a given date ($P = 0.03$) best explained the variation in milk weight on farm. The relationship between milk weight and logSCC was negative; therefore, as logSCC increases, milk weight per farm decreases by 10.9 kg (se = 5.2). As horn flies per farm increases, overall milk weight was found to decrease:

Milk Weight = 74.0- 0.03 (average horn fly numbers)

Discussion

An increase in logSCC was found to be associated with an increase in DIM (Figure 2.2). Previous research has shown that late-lactation cows are more likely to develop or maintain a high SCC (Braund and Schultz, 1963, Olde Riekerink et al., 2007). Our results showing a 0.3 logSCC increase, or a 33% increase, with every 100 DIM are in agreement with previous studies. Hagnestam-Nielsen and others determined multiparous cows free from clinical mastitis in 9 to 16 weeks of lactation had a median SCC of 51,000 cells/mL; whereas multiparous cows in weeks 33 to 44 of lactation had a median SCC of 164,000 cells/mL (Hagnestam-Nielsen et al., 2009). This study found that cows experience a 31% increase in SCC during the same time frame. An increase in SCC with DIM can be caused by response to infection, as well as an increased concentration due to declining milk yield. The interaction of DIM and parity can also affect SCC (Wiggans and Shook, 1987). Older cows that are late in lactation typically have higher SCC, which can also be supported by this study. In our study, cows in 4th or greater parity and over 250 DIM, had a higher mean SCC compared to cows that were in 1st, 2nd or 3rd parity and in late

lactation (925,187 cells/mL and 372,747 cells/mL, respectively). DIM continuously increased throughout our study (Table 2.3).

Somatic cell count was not significantly associated with season when all farm were included ($P = 0.5$), which differs from previous studies (Bishop et al., 1980, Hogan et al., 1989, Olde Riekerink et al., 2007, do Amaral et al., 2011, Shock et al., 2015). The largest peaks in SCC were found in the months of July, August, and September, but varied largely by farm (Table 2.1). When the model was broken out by farm, season was only associated with logSCC on farm 3 ($P = 0.04$). The variation in logSCC between farms and between cows within an individual farm could explain why no relationship was found between logSCC and horn fly numbers. The lack of a relationship between horn fly numbers and logSCC is similar to the findings in pastured beef cattle (Mays et al., 2014).

In our study, when testing the effects on milk weight, logSCC was negatively correlated with milk weight—both at the cow and herd level—so with every one unit increase in logSCC milk yield is expected to decrease by 1.4 kg (se = 0.5) and 10.9 kg (se = 5.2), respectively. The natural log of SCC has previously been correlated with milk yield such that a cow with relatively high SCC (250,000 cells/mL) compared to a cow with a relatively low SCC (50,000 cells/mL) produces on average, 1.6 kg/d less milk (Potter et al., 2018). An increase in THI during the summer months also can have an impact on milk weight. Previous studies determined that daily THI is negatively correlated to milk yield (r = -0.76) (Bouraoui et al., 2002). In the aforementioned study, when THI increased from 68 to 72, milk production decreased by 21%, with maximum reductions observed when THI reaches 80 or above (Bouraoui et al., 2002). The average THI in this study was 84.4 (± 7.5), but THI was not associated with a reduction in milk weight ($P = 0.32$) in our multivariable model. This finding could reflect the statistical model

where we used a single THI value per farm on a collection date, not the level of heat stress experienced by individual cows. Unfortunately, labor and time constraints precluded us from collecting this additional data. When the model was broken out by farm, THI was a significant factor associated with milk weight on farms 2, 3, and 4 ($P = 0.004$; $P = 0.003$; $P = 0.05$; respectively). THI tended towards a low correlation with horn fly numbers ($r = 0.1$; $P = 0.1$).

Horn fly numbers per cow had a significant negative effect on milk weight (Figure 2.4). Mays and colleagues (2014), also found a negative relationship between horn fly numbers and milk yield in beef cattle. Milk yield decreased by 0.72, 0.68, and 0.71 kg/d per unit increase in log horn fly count in May, June, and July, respectively (Mays et al., 2014). Dairy cows naturally produce more milk than beef cows, potentially explaining the larger loss in milk yield for our study. In contrast, Wolley and others (2018), did not find a relationship between milk weight and horn fly numbers (Woolley et al., 2018). Dairy cows in that study were treated with essential oils every other week, for a total of 9 weeks, to determine behavioral and physiological responses to flies (Woolley et al., 2018). With the alternating application of essential oils, cows were allowed a recovery time where horn fly numbers decreased when essential oils were applied. The inconsistent exposure to horn flies could lessen the overall impact of fly pressure and subsequent relationship with milk yield. This study was also conducted in Canada, where the summers are milder than the southeastern United States and may lessen the individual and/or combined influence of heat stress and fly pressure on milk yield.

When using equation 2 described in the results, once a cow reaches a 100 horn flies, milk yield is expected to decrease by 1 kg/milking at the cow level and 3 kg/milking at the herd level if the herd averages 100 flies per cow. If we use the conservative estimate of cows losing approximately 1 kg of milk per milking with every increase in 100 horn flies, producers that are

not controlling horn flies are decreasing profitability during the summer months. Assume an organic dairy farm is milking 100 Jersey cows twice a day and the cows are producing an average of 20 kg of milk per day. With the average price of $31.30 /cwt for organic milk in the year 2019, and assuming each individual cow in the herd has 100 horn flies, the producer can expect to lose $138 per day. The economic injury threshold, which is defined as the pest density at which action should be taken to prevent a pest population from reaching the economic injury level, is an important factor here. Economic injury level is then defined as the smallest number of insects that will cause yield losses equal to the insect management costs. Producers can now associate the reduction in milk yield during the summer months to an increase in horn fly numbers, which may be controlled. The producer can determine how much money and time is spent in controlling horn fly populations in order to stabilize milk weights during the summer.

Increases in horn fly numbers during the summer months follow similar trends shown in previous research (Maldonado-Siman et al., 2009). Increases in horn fly numbers are moderately correlated with relative humidity, rainfall, and average temperature (Lima et al., 2003, Maldonado-Siman et al., 2009). According to Morgan (1964), the ideal environment for horn fly survival is temperature of 23 to 27 °C, relative humidity ranging from 65 to 90%, scattered light rain showers, and no wind (Morgan, 1964). The range of temperature during the summer months in this study was a low of 21°C and a high of 29°C. Relative humidity ranged from 57 to 86%. The combination of an increase in temperature and humidity throughout the study likely explains the increase in horn fly populations.

Although not a component of this study, we can speculate the variation in horn fly numbers on an individual farm are due to natural infestation numbers, environmental features including abiotic and biotic variables, as well as the control measures put in place. Farm 4 had

the lowest number of horn flies and used fly tape that ran the length of the tiestall barn, applied essential oils to the cows every 3 days, and housed the cows in a tunnel-ventilated tiestall barn during the heat of the day. The average wind speed in the tunnel-ventilated barn on Farm 4 was 11 mph. According to Morgan (1964), horn flies prefer an environment with no wind so it can be speculated that sustained winds could reduce horn fly numbers regardless of other ideal environmental conditions. Of particular note, this farm was the only one in which horn fly numbers were not associated with reductions in milk yield. In contrast, Farm 3 had the largest population of horn flies and only applied essential oils to cows once a week to control fly populations. Essential oils have been found to repel > 75% of the horn flies on the treated area for up to 6 hours on pastured cattle (LaChance and Grange, 2014). With Farm 3 only applying essential oils to the cows once a week, it was not an effective control measure for horn flies. Although not statistically compared, control strategies used on these two farms could start to explain the importance of fly control measures and their effectiveness.

Conclusion

The results from this study establish the relationship between horn fly numbers, SCC, and milk yield for organic dairy farms in the southeast United States. Horn fly numbers continuously increased throughout the summer. No association was found between the increase in horn flies and SCC. A reduction in milk yield, though, was found with an increase in horn fly numbers. Horn fly number, when considered in combination with the DIM, parity, logSCC, and THI, was the most relevant factor explaining a reduction in milk weight during the summer months. This study emphasizes the importance of fly control during the summer months. In order to maintain milk weight during the summer months, effective fly control measures should be implemented.

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CHAPTER III

Staphylococcus Mastitis Pathogens are Present in Milk and Horn Fly Populations

Abstract

Prevention and treatment of mastitis without the use of antibiotics or synthetic products is one of the challenges organic dairies face. Greater understanding of the factors affecting mastitis will aid in developing management programs. Mastitis and fly populations both increase during the summer months, but the relationship between the two is unknown in lactating dairy cows housed partly or fully on pasture. Our objective was to identify specific *Staphylococcus* mastitis pathogens (*Staph. aureus, Staph. chromogenes*, *Staph. hyicus,* and *Staph. agnetis*) in quarter milk samples and horn fly populations. Four organic dairies were enrolled in the study and visited May through October 2019. Aseptic quarter milk samples were collected, regardless of somatic cell count (SCC) or clinical status, from the entire lactating herd once a month, cultured, and *Staph*. isolates were identified using multiplex PCR. Live flies from the dorsal midline and ventral midline area were collected from 15 focal cows on each herd (n = 65 cows) every 2 weeks. Flies were pooled by farm, date, cow, dorsal midline or ventral midline, and sex with a max of 15 flies and minimum of 1 fly per pool. DNA was extracted from whole flies using a QIAcube HT and specific *Staph*. species were determined in the fly by multiplex PCR and visualized using a QiAxcel. Multivariable logistic regression with generalized linear mixed models (PROC GLMMIX) was used to determine cow-level, herd-level, and horn fly pool risk factors associated with each to test positive for the *Staph.* species. A horn fly pool which tested positive for *Staph. chromogenes* had an effect on both a cow and herd to test positive for *Staph. chromogenes* ($P = 0.02$, $P = 0.02$; respectively). Horn fly pools collected off the dorsal midline had a lower probability of testing positive for *Staph. aureus* ($P = 0.001$), *Staph. agnetis* ($P = 0.002$), and *Staph. chromogenes* ($P = 0.07$), versus those collected off the ventral midline area. Female horn fly pools were more likely to test positive for *Staph. aureus* ($P = 0.003$),

Staph. chromogenes ($P = 0.02$), and *Staph. agnetis* ($P < 0.0001$) than male horn fly pools. The results from this study determine common *Staphylococcus* mastitis pathogens present in milk samples, are also commonly found in horn fly populations on organic dairy farms in the southeast United States.

Introduction

Mastitis, an inflammation of the mammary gland, is the most common disease found on dairy farms in the United States. The type of pathogen causing the infection influences the severity and duration of mastitis, but the most frequently isolated organisms from dairy cattle mammary glands causing mastitis are *Staphylococcus* (Hogan et al., 1989, Levison et al., 2016). The most common *Staphylococcus* species isolated include *Staph. aureus* and *Staph. chromogenes*, while *Staph. agnetis*, and *Staph. hyicus* are less prevalent. Briefly, *Staph. aureus* is a contagious, gram-positive cocci that can be isolated from various areas of the farm such as housing, feed, other animals, water, and insects (Roberson et al., 1994, Gillespie et al., 1999, Anderson et al., 2012). Of the coagulase negative Staphylococci (CNS) species identified, the most frequently isolated species is *Staph. chromogenes* (Sampimon et al., 2009, Supré et al., 2011). *Staph. chromogenes* causes persistent infections and is associated with a significant increase in SCC, but has a minimal effect on milk production (Supré et al., 2011, Fry et al., 2014, Moroni et al., 2018). *Staph. hyicus* is a coagulase-variable *staphylococcal* species that is part of the commensal flora of various animals and is commonly isolated from bovine milk (Trinidad et al., 1990, Gillespie et al., 2009). When differentiating *Staph. hyicus* using the API ® Staph method, which employs biochemical testing, can be misidentified (Adkins et al., 2017). The overall prevalence of *Staph. agentis* on farm was low, ranging form 0.0% to 2.2% (Adkins et al., 2017).

Mastitis also has been found to increase during the summer months. Hogan et al (1989), found that clinical mastitis is highest during the summer months, while decreasing throughout the fall and winter months (Hogan et al., 1989). Subclinical infections are identified by elevated somatic cell count (SCC), or the total number of cells/mL in milk. The SCC are primarily

composed of leukocytes produced by the cow's immune system to fight infection and cows with a SCC >200,000 cells/mL are considered to have a subclinical infection. When observing seasonality changes in herd bulk milk somatic cell count (BMSCC), individual cow SCC and incidence rate of clinical mastitis, all were found to increase during the summer, with BMSCC count peaking in August to September (Olde Riekerink et al., 2007, Rowbotham and Ruegg, 2015). With summer comes an increase in heat and humidity that provide optimal environmental conditions for bacterial growth. Heat stress also causes immune dysfunction in cows, increasing the risk of intramammary infections (do Amaral et al., 2011).

The increase in mastitis also may be associated with an increase in horn fly (*Haematobia irritans*) populations during the summer months. Horn flies can carry *Staph. aureus,* which has been found to cause summer mastitis in heifers or young female cattle prior to having their first calf (Nickerson et al., 1995). A common *Staph. aureus* subtype was found in both heifer and fly isolates suggesting horn flies play a role in the transmission of *Staph. aureus* to heifers (Gillespie et al., 1999). In a similar study, 55.8% of horn flies tested positive for *Staph. aureus;* whereas, 13% and 17% tested positive for *Staph. aureus* in multiparous cow milk samples and primiparous cow colostrum samples, respectively (Anderson et al., 2012). These studies suggest that flies and heifer body sites could be an important source of *Staph. aureus* for heifer intramammary infections. *Staph. aureus* also was the most abundant bacteria in the adult female horn fly among 25 OTUs (Operational Taxonomic Unit) identified in horn flies (Palavesam et al., 2012). In adult dairy cows, the use of fly control also has reduced the amount of *Staph. aureus, coagulase negative staphylococci* and *Escherichia coli* intramammary infections (Arsenopoulos et al., 2018). It is known that on dairy farms, both mastitis and fly populations exists during the summer months, but the relationship between the two is unknown in lactating

dairy cows housed on pasture in the southeastern United States. This region has extended periods of heat and humidity during the summer months. This study tests the hypothesis that horn flies carry mastitis-causing pathogens and, in result, cows will have an increase in *Staph.* infections.

Materials and Methods

This study was conducted on four USDA certified organic dairy herds located in Tennessee and Kentucky. Participating in regular Dairy Herd Improvement (DHI) testing (DHIA, Knoxville, TN; Mid-South Dairy Records, Springfield, MO) was a requirement for all farms. The University of Tennessee and University of Kentucky Extension Cooperatives were used to recruit each farm. The University of Tennessee Institutional Animal Care and Use Committee approved all animal- related procedures for this study (Protocol Number 2391). Sampling occurred during the months of May through October 2019.

Animals and Management

Fifty-five (SD ± 17) lactating cows was the average herd size for the four farms. Individual herds consisted of Holstein, Jersey, or cross-bred cows. Milking occurred twice daily on each farm, with morning milking occurring between 0500 and 0700h and evening milking occurring between 1700 and 1900h. To minimize the effects of clinical mastitis and swelling associated with calving on all farms, peppermint-based udder cream was used as needed on all farms. No other treatments were used to treat clinical mastitis on any farm.

When cows were not on pasture, housing included tiestalls, compost bedded packs, or concrete bedded pens. Farm 1 housed cows in concrete pens, whereas Farms 2 and 3 housed cows in compost bedded packs. A tunnel ventilated tiestall barn was used on Farm 4 and housed cows in it during the heat of the day. All herds had access to pasture and was relied on for more than 30% of the cows dry matter intake, as required by the USDA organic certification. Grazing

occurred at the minimum through the months of April through October, with the requirement that grazing must be ≥ 120 d.

To control for flies on farm, Farm 1 used a CowVac (Spalding Laboratories, Reno, NY) twice a day at the time of milking. Farm 2 used fly predators twice a month starting in April through the end of October and applied essential oils designed for fly control to the cows twice a week. Weekly application of essentials oils was the only fly control method for Farm 3. Fly tape, running the length of the tiestall barn, and application of essential oils to the cows every 3 days was used as fly control on Farm 4.

Data Collection

THI Data. Temperature and humidity for each sample date was accessed through an online database (Weather Underground, www.wunderground.com). To determine THI from the temperature and humidity data collected, the subsequent equation was used (Ravagnolo et al., 2000); T= air temperature (°C) and RH = relative humidity (%):

$$\mathbf{THI= (1.8T + 32) – [(0.55 - 0.0055RH) x (1.8T - 26)]}$$

Sample dates were also characterized into season according to the astronomical definition, with spring beginning on March 21st, summer beginning on June 21st, and fall beginning on September 21st. Spring included 1 sample date for each farm, summer included 3 sample dates, and fall included 1 sample date; the exception was farm 4 who discontinued from the study at the end of August.

Milk Data. To collect an individual cow's SCC and milk weight, DHI tests were used. Testing occurred on average every 27 (± 5) days on each farm for the entire lactating herd, resulting in 6 collection dates. With Farm 4 leaving the study at the end of August, it only had 4 DHI collection dates. Milk samples from Farms 2, 3, and 4 were analyzed in the DHIA lab at the

University of Tennessee (Knoxville, TN); whereas milk samples from Farm 1 were collected and analyzed through Mid-South Dairy Records (Springfield, MO).

Aseptic milk collection. Following the National Mastitis Council guidelines (Oliver et al., 2004), aseptic milk samples were collected from all functional mammary quarters on the entire lactating herd on each farm and assessed for microbial presence. Sampling occurred every 28 days for a total of six (6) collection dates. Milk samples were frozen following visits until further processing could occur. Organisms were identified through the Tennessee Quality Milk Laboratory (Knoxville, TN). Ten μL of milk from each quarter was plated on a quadrant of Trypticase soy agar with 5% sheep blood (BD, Sparks, MD). The plates were incubated at 37°C and were observed for 3 d, with 24 h increments. Morphological features, catalase tests, and Gram staining were used to identify the bacteria. *Staphylococci* species were further tested for coagulase using the tube coagulase method. To determine the *Staphylococcus* species, the API (Analytical Profile Index) Staph System (bioMerieux Inc., Hazelwood, MO, USA) was used. Quarters were considered positive for intramammary infections if samples had a total of 1 or 2 organisms isolated. Samples were considered contaminated when more than 3 organisms were identified or contained *Bacillus*.

Stock cultures were created by streaking ten μL of milk from each quarter on Trypticase soy agar with 5% sheep blood (BD, Sparks, MD). The plates were incubated at 37°C and were observed for 3 d, with 24 h increments. Once adequate growth was observed, multiple colonies were removed from the plate using a lawn sweep, placed in 5 mL of Tryptic Soy Broth (TSB), and incubated at 37°C overnight. Five hundred μL of the enriched colonies were removed the following morning and placed in 500 μL of glycerol. Cryovials were placed in -80°C freezer until further processing could occur.

Stocked cryovials that had previously been identified as having *Staph. aureus*, *Staph. hyicus*, *Staph agenetis*, and *Staph. chromogenes* using the API Staph System were then confirmed using PCR. Ten µL of the stock culture was inoculated in 1.5 mL of TSB broth and placed in a shaking incubator at 200 rpm at 37°C overnight. After approximately 14 h of incubation, the 96 deep well plate was centrifuged at 858 g for 1 hour to produce pellet. Supernatant was removed and the pellet was placed in -80°C freezer until further processing could occur.

Capturing Individual Animal Fly Counts. To determine horn fly number for individual cows on each herd, 15 focal cows from each herd (n = 65) were used. Cows were selected based on stage of lactation so the cow would remain in the study throughout the entire sampling period. The mean (± SD) for stage of lactation was 187 (± 65), whereas parity, which is the total number of lactations the cow has had, was 4 (± 2). If cows were dried off in order to prepare for the next calving or removed permanently from the herd, replacement cows were added to the group on the following visit to keep the focal number at 15 cows per visit. A Nikon Coolpix P1000 16.0-Megapixel Digital Camera (Nikon, Tokyo, Japan) was used to take pictures of the flies present on an individual cow's dorsal midline and ventral midline area. Each focal cow had a total of two pictures each visit. Then, using these pictures, convolutional neural network was used to segment the salient cow in each image. A separate network was then used to locate the horn flies and provide a total fly count for that area in the picture (Psota, unpublished). To get the total number of horn flies on each individual focal cow on a given date, the number of flies on dorsal midline and ventral midline were added together. Pictures were taken of individual cows every 12.8 d (± 5) on each farm, for a total of 12 visits; except for Farm 4 who had 8 visits total.

Horn fly collection. The same 15 focal cows were used to collect live flies from both the dorsal midline and ventral midline area every other week. Horn flies from the ventral midline were collected while cows were in the parlor or tiestall for milking, whereas flies from the dorsal midline were collected after cows exited the milking parlor or while in the tiestall. Mesh nets were used to collect the flies and were disinfected between farms by spraying each net with 70% ethanol. After flies were collected in the mesh nets, they were transferred to 2 oz. Nasco Whirl-Pak Bags and placed directly on ice. Flies were frozen until further processing could occur.

Frozen flies in each Whirl-Pak bag were then identified to species and sex. Counted and sorted horn flies were placed into new Whirl-Pak bag using sterile forceps to create pools that included farm, date, cow, dorsal midline or ventral midline, species, and sex. The average number of flies in each pool was 4.1 (± 3.9), with the minimum being 1 fly and a maximum of 15 flies. Forceps were sterilized with 70% ethanol between each pool. Each of the pools were transferred to a 96- collection microtube rack that contained one 3 mm bead per microtube. Plates remained frozen until further processing could occur.

Staph. species identification. DNA extraction was performed on milk sample pellets and each fly pool in the QIAcube HT using the QIAamp 96 DNA kit (Qiagen, Germantown, MD). One hundred eighty μL of animal tissue lysis (ATL) buffer and 20 μL of proteinase K was added to the milk sample pellets and 280 μL of ATL buffer and 20 μL of proteinase K was added to each well that contained the fly pool and bead according to the manufacturer instructions. Racks of collection microtubes containing the flies were put into the TissueLyser for 30 seconds at 15 Hz to lyse flies. Following lysing, microtubes were centrifuged at 858 g for 10 seconds. Both the fly samples and milk sample pellets were placed in incubator at 130 rpm and 56°C overnight.

The following morning, the samples were transferred to an S block and DNA was extracted using the QIAcube HT (Qiagen, Germantown, MD) according to the manufacturer instructions.

Primers for the PCR reactions included *Staph. aureus*, *Staph. hyicus*, *Staph. agnetis*, and *Staph. chromogenes* (Table 3.1). For the milk sample pellets and horn fly pools, 12.5 µL of Platinum II Hot Start Master Mix (Invitrogen, Carlsbad, CA), 0.5 µL of each 100µM primer, 6.5 µL of sterile water, and 2 µL of DNA, for a total of 25 µL, was added to each well in a 0.3 mL 96-well PCR plate. To determine specificity of the primers, both positive and negative controls were used. For each *Staph.* species, a pre-PCR step was run at 94°C for 15 minutes, followed by 35 cycles under the following conditions: denaturing at 94°C for 30 seconds, annealing at 55°C for 30s, and extension at 72°C for 1 min. After the final cycle, the samples were held at 72°C for 5 minutes to complete the reaction and held at 4°C. Samples were then visualized in the QiAxcel (Qiagen, Germantown, MD) using the QiAxcel DNA screening kit (Qiagen). QX DNA size marker 50-800 bp, QX alignment marker 15 bp/ 1 kb, and the AL420 method was used to screen the DNA as recommended by the Qiaxcel DNA Handbook. Samples were visualized using Qiagen Biocalculator.

Statistical Analysis

If a cow (n = 16) entered the study with a SCC > 200,000 cells/mL, the cow was removed from the analysis because a starting SCC > 200,000 cells/mL indicates a cow may have a subclinical infection present and inadequately represents the horn fly's association with mastitis. When the cow recovered and SCC was ≤ 200,000 cells/mL, the cow (n = 6) was added back into the analysis and remained there for the rest of the study.

Cows were also removed from the cow-level (n = 5) and herd-level (n = 37) analysis if one or more of the cow's quarters consistently had one of the *Staph.* species present throughout

the entire study. If a cow entered the study with a *Staph*. species present in any quarter, but cleared the infection by the subsequent visit, that cow was removed from the cow-level (n = 4) and herd-level (n = 4) analysis for the first sample date only. A cow beginning the study or having a chronic infection throughout the study would inaccurately represent the horn fly's association with a cow testing positive for a *Staph.* species.

To determine cow-level risk factors associated with the probability for a cow to test positive for *Staph. aureus*, *Staph. chromogenes*, *Staph. hyicus*, or *Staph. agnetis*, multivariable logistic regression with generalized linear mixed models (PROC GLIMMIX) within SAS 9.4 (Cary, NC) was used. The fixed effects included THI, season, DIM, parity, milk weight, total number of flies per cow on a given date, horn fly pools that tested positive for the same *Staph*. species, and the interaction of total number of horn flies*fly pool testing positive for the same *Staph.* species. Random effects included farm and cow(farm) and a repeated measure of date. Backward elimination was used to develop the most parsimonious informative model. Fixed effects were removed if the F-statistic was < 1.0. Of the fixed effects tested, season, DIM, and milk weight were not retained in the final model for any of the *Staph.* species. Significance was considered at $P < 0.05$ and a trend at $P < 0.1$.

Multivariable logistic regression with generalized linear mixed models (PROC GLIMMIX) within SAS 9.4 (Cary, NC) was used to determine herd-level association between fly pressure variables in and the portion of cows in the herd testing positive for *Staph. aureus*, *Staph. chromogenes*, *Staph. hyicus*, or *Staph. agnetis*. Fixed effects included average total number of flies of the 15 focal cows on a given date for each herd and the average number of horn fly pools that tested positive for the same *Staph*. species as the response variable. To obtain the proportion of cows positive for a single *Staph.* species, a binary variable was created where

absence was 0 and presence in any of the lactating quarters for a cow was recorded as 1. The herd mean probability was then calculated to provide the final herd level response variable. Random effects included farm and a repeated measure of date. Significance was considered at $P \leq 0.05$ and a trend at $P < 0.1$.

To determine the factors associated with a fly pool testing positive for *Staph. aureus*, *Staph. chromogenes*, *Staph. agnetis*, and *Staph. hyicus*, multivariable logistic regression models (PROC GLIMMIX) within SAS 9.4 (Cary, NC) was used. Fixed effects included whether the horn fly was collected off the dorsal or ventral midline, sex of the pool, and total number of flies per cow on a given date. Random effects included farm and cow(farm), with a repeated measure of date. Significance was considered at $P \leq 0.05$.

Results

Staph. species in milk. Out of the 15 focal cows on each farm throughout the study, 9.0% tested positive for *Staph. aureus* in at least one quarter during the sampling period, 7.2% for *Staph. chromogenes*, 0% for *Staph. hyicus*, and 4.2% for *Staph. agnetis*. When compared to overall herd prevalence, 9.2% of all lactating cows on each farm tested positive for *Staph. aureus* in at least one quarter during the sampling period, 10.2% for *Staph. chromogenes*, 0% for *Staph. hyicus*, and 4.1% for *Staph. agnetis*. Farm 2 had the greatest prevalence of each bacteria throughout the study, with 18.2% of cows testing positive for *Staph. aureus*, 19% testing positive for *Staph. chromogenes*, and 1.7% testing positive for *Staph. agentis* in at least one quarter throughout the study.

Initial analyses examined cow and environment risk factors tied to presence of targeted *Staph*. species in milk of focal cows (Table 3.2). A horn fly pool that was positive for *Staph. chromogenes* was significantly associated with a cow testing positive for *Staph. chromogenes* (*P*

= 0.02). In contrast, no factors were associated with a cow testing positive for *Staph. aureus* or *Staph. agnetis*. We then used the mean fly pressure and percent of fly pools positive for individual *Staph.* species collected from the focal cows as estimates for the herd to evaluate these effects at the farm or herd level. Similar results were obtained at the herd level. When herd level associations were determined for *Staph. aureus*, *Staph. chromogenes*, and *Staph. agnetis*, a horn fly pool testing positive for *Staph. chromogenes* had a significant association with a herd testing positive for *Staph. chromogenes* (P = 0.02; Table 3.3). No factors were associated with a herd testing positive for *Staph. aureus* or *Staph. agnetis*.

Staph. species and horn flies. When determining bacteria found on or in the horn flies, 48.7% of the horn fly pools tested positive for *Staph. aureus*, 34.0% for *Staph. chromogenes*, 8.8% for *Staph. hyicus*, and 39.4% for *Staph. agnetis*. Twenty-six percent of horn fly pools were free from bacteria species, 34% had 1 bacteria species present, 28% had 2 bacteria species present, and 12% had 3 bacteria species present. The most common combination of bacteria found in the horn fly pools was *Staph. aureus* and *Staph. agnetis* at 14%, while 10% of the horn fly pools had a combination of *Staph. aureus*, *Staph. chromogenes*, and *Staph. agnetis* present.

Of all the horn fly pools collected for the 15 focal cows, 43.7% were collected off the dorsal midline (n = 220) and 56.3% were collect off the ventral midline (n = 283). Differences were observed in the type of bacteria found in the horn fly pools depending on location of collection (Table 3.4). Dorsal midline or ventral midline collection area was a significant factor associated with a fly pool testing positive for *Staph. aureus* (P = 0.001) and *Staph. agnetis* (P = 0.002), while trending for *Staph. chromogenes* (P = 0.07). Horn fly pools collected off the dorsal midline had a lower probability of testing positive for *Staph. aureus* (OR: 0.5; 95% CI: 0.4, 0.8), *Staph. agnetis* (OR: 0.5; 95% CI: 0.4, 0.8), and *Staph. chromogenes* (OR: 0.7; 95% CI: 0.5, 0.1),

versus those collected off the ventral midline area. Interestingly, female horn flies also were 1.4 times more likely to be caught off the ventral midline area when compared to male horn flies (OR = 1.4; 95% CI: 1.2, 1.7).

The sex of the horn fly pool also was a significant factor associated with a fly pool testing positive for *Staph. aureus* ($P = 0.003$), *Staph. chromogenes* ($P = 0.02$), *Staph. agnetis* ($P < 0.0001$) and *Staph. hyicus* ($P < 0.0001$). Female horn fly pools were more likely to test positive for *Staph. aureus* (OR: 1.8; 95% CI: 1.2, 2.6), *Staph. chromogenes* (OR: 1.6; 95% CI: 1.0, 2.4), and *Staph. agnetis* (OR: 3.5; 95% CI: 2.3, 5.3) than male horn fly pools (Table 3.5). In contrast, female horn fly pools were less likely to carry *Staph. hyicus* compared to male horn flies (OR: 0.09; 95% CI: 0.04, 0.2).

Discussion

The prevalence of each tested bacteria, both in the 15 focal cows and the entire lactating herd on each farm, compares similarly to previous research. *Staph. aureus* was found to be present in at least one quarter for 9.0% of the 15 focal cows on each farm and 9.2% of all lactating cows on each farm in this study. Previous studies, when observing the prevalence of mastitis pathogens, determined that *Staph. aureus* was one of the most prevalent isolates cultured in milk and heifer colostrum samples (Østerås et al., 2006, Anderson et al., 2012). Østerås and others (2006), also determined that *Staph. aureus* infections have the highest prevelance from May to July (Østerås et al., 2006), which is also when horn fly populations increase. *Staph. chromogenes*, in this study, was found to be present in at least one quarter for 7.2% of the 15 focal cows on each farm and 10.2% of all lactating cows on each farm. Previous research has determined that the most frequently isolated CNS species is *Staph. chromogenes* (Sampimon et al., 2009, Supré et al., 2011). Four percent of the 15 focal cows and 4.1% of the entire lactating

herd on each farm testing positive for *Staph. agnetis*, is in agreement with a recent study by Adkins and others, that determined the overall prevalence of *Staph. agentis* is low, ranging from 0.0% to 2.2% (Adkins et al., 2017).

Although not a direct question of this study, we tentatively confirmed the findings of previous research indicating classification errors when using biochemical testing (Park et al., 2011, Adkins et al., 2017). All of the *Staph. hyicus* present in milk samples previously identified using the API Staph System (n = 11) were subsequently identified as *Staph. aureus*, *Staph. chromogenes*, or *Staph. agnetis* when using a multiplex PCR. Adkins and others (2017) found similar results when testing isolates previously identified as *Staph. hyicus* using coagulase testing and API Staph, with 42 coagulase positive isolates identified as *Staph. agnetis,* 8 coagulase negative isolates identified as *Staph. chromogenes*, and 5 isolates identified as *Staph. aureus* when using housekeeping gene sequencing and multiplex PCR (Adkins et al., 2017). We stated tentative confirmation, as the initial species determination via API Staph was conducted on a single colony, whereas follow-up testing was conducted using a lawn sweep, collecting multiple colonies from the entire plate, while also enriching the bacteria immediately prior to multiplex PCR. The findings from these studies support the idea that characterizing certain bacteria isolates on phenotypic identification methods alone can lead to classification errors. We also must consider the sampling process. Initial API Staph diagnostic assays are performed on a single colony, selected from a potentially larger pool of colonies on the original plate that look similar at a macro level. Thus, other species could be present. The identification of multiple species present in milk by multiplex PCR can provide a more accurate and comprehensive representation of bacteria present in the milk collected from a cow's mammary gland.

A relationship was found in both a cow ($P = 0.02$) and herd ($P = 0.02$) testing positive for *Staph. chromogenes* when a horn fly pool also tested positive for *Staph. chromogenes*. No relationship was found with either *Staph. aureus* or *Staph. agnetis*. *Staph. chromogenes* is commonly found on the skin surface (De Vliegher et al., 2003, Anderson et al., 2012, da Costa et al., 2014) where horn flies would be able come in contact and potentially transfer it to the teat end where it could internalize in the mammary gland. However, little is known about the transmission of *Staph. chromogenes* from the environment to the mammary gland. *Staph. aureus,* on the other hand, is more contagious in nature than *Staph. chromogenes*, with contaminated milk being the primary driver of infections in lactating cows by transfer via milking unit liners and milking personnel themselves. This suggests *Staph. aureus* may commonly be transferred between cow to cow, whereas *Staph. chromogenes* is more commonly transferred from the environment.

In this study, our primary question was regarding the prevalence of infection relative to fly pressure. This approach would not be able to determine the degree to which specific flies transmit specific strain types of each *Staph.* species as has been demonstrated in other research. Due to potential movement of flies between heifers and multiparious cows, Gillespie and others (1999) compared the strains of *Staph. aureus* found in horn flies collected off heifers and strains found in mammary secretions from multiparious cows and did not find a relationship; although they did find a direct relationship between the *Staph. aureus* strains in horn flies collected off the heifers and mammary secretions of the heifers (Gillespie et al., 1999). In contrast, a similar *Staph. aureus* genotype was found when isolating *Staph. aureus* from milk and colostrum samples, heifer body sites, and horn flies (Anderson et al., 2012). The prior studies focused on the genetic relatedness of *Staph. aureus* identified in dairy cattle versus horn flies to establish the

potential for horn flies as a source for intramammary infections. In contrast, our focus was to determine the association between the percentage of horn flies testing positive for each *Staph.* species and the percentage of cows testing positive for the same *Staph.* species. Further research determining if the strains of specifically *Staph. aureus* and *Staph. chromogenes* are the same between horn fly pools and milk samples could provide further insight to the sources and nature of intramammary infections promoted by the presence of horn flies.

Despite the lack of enrichment, 48.7%, 34.0%, and 39.4% of horn flies were positive for *Staph. aureus*, *Staph. chromogenes*, and *Staph. agnetis*, suggesting flies carry higher loads of bacteria than milk which often requires enrichment for detection. It is not surprising that horn flies commonly carried *Staph. aureus* and *Staph. chromogenes*, which were also the predominant organisms found in the milk samples and are commonly found on skin surfaces (Haveri et al., 2008, Anderson et al., 2012, da Costa et al., 2014). With no milk samples testing positive for *Staph. hyicus*, it would be expected that horn flies also had a reduction in the presence of *Staph. hyicus*. The horn flies may also pick up the *Staph.* species that reside on the skin near the udder area, as hair loss and an increase in skin exposure was observed in that area. Further research is needed to determine if the *Staph.* species found on the horn fly is the same strain as the *Staph.* species found in the milk samples.

Horn flies have previously been found to carry *Staph. aureus* (Chirico et al., 1997, Gillespie et al., 1999, Anderson et al., 2012, Palavesam et al., 2012), but limited research is associated with horn flies carrying *Staph. chromogenes*, *Staph. agentis* and *Staph. hyicus*. Female horn flies were more likely to carry *Staph. aureus*, *Staph. chromogenes* and *Staph. agnetis* (OR: 1.8; 95% CI: 1.2, 2.6; OR: 1.6; 95% CI: 1.0, 2.4; OR: 3.5; 95% CI: 2.3, 5.3, respectively), but less likely to carry *Staph. hyicus* (OR: 0.09; 95% CI: 0.04, 0.2; Table 3.5).

With female horn flies carrying more *Staph.* species and more likely to be caught off the ventral midline, could explain why fly pools collected off the ventral midline carried more *Staph.* species. It must also be noted that more female pools were collected and typically had more females present in the pool. Having more horn flies present in female horn fly pools could bias the results. To address this we removed pools that had more than 5 horn flies present and confirmed the original results of the analysis. The relationship also held true regardless of the number of female horn flies and total number of horn flies within the pool. Palavesam and others (2012) also determined that *Staph. aureus* was the most abundant bacteria in the adult female horn fly (Palavesam et al., 2012). It was also determined that adult male horn flies carry *Staph. hyicus,* but was not associated with female horn flies (Palavesam et al., 2012). During collections, it was also observed that the ventral midline had less hair present than the dorsal midline, potentially exposing the horn flies to more of the normal flora *Staph.* bacteria residing on the skin.

Conclusion

The results from this study determine common *Staphylococcus* mastitis pathogens present in milk samples, can also be found in horn fly populations on organic dairy farms in the southeast United States. *Staph. aureus*, *Staph. chromogenes,* and *Staph. agnetis* were all found to be present in both milk samples and horn fly pools, with *Staph. hyicus* present in horn fly pools alone. A Staph. chromogenes-positive horn fly pool had a significant association with a cow and herd to test positive for *Staph. chromogenes*. Female horn flies are more likely to carry *Staph. aureus*, *Staph. chromogenes,* and *Staph. agnetis* and are more likely to be caught off the ventral midline area than male horn flies. This suggests female horn flies could be the source of *Staph.* transmission in ventral midline area. Further research is needed to determine if the same *Staph.*

strain was present in the milk and horn fly samples to strengthen the relationship between the two.

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CHAPTER IV
Conclusions

Mastitis is the most common disease found on dairy farms in the United States and summer mastitis, specifically, continues to be a challenge on many dairy farms. Organic farms have the added challenge of treating and preventing mastitis without the use of antibiotics or synthetic products. Understanding factors associated with the probability of mastitis will aid in developing mastitis control programs. Limited research is available on determining the association between horn flies, milk quality, milk yield, and *Staphylococcus* mastitis on organic dairy farms. This study 1) determined the association between horn fly numbers, SCC, and milk yield on organic dairy herds and 2) identified specific *Staphylococcus* mastitis pathogens (*Staph. aureus*, *Staph. chromogenes*, *Staph. hyicus* and *Staph. agnetis*) in quarter milk samples and horn fly populations.

Horn fly numbers continuously increased throughout the summer. Unexpectedly, an association between horn fly numbers and SCC was not found. Horn flies, when combined with the other factors of DIM, parity, logSCC, and THI, was the most relevant to a reduction in milk weight during the summer months. We found that for every increase in 100 horn flies, milk is expected to decrease by 0.99 kg/ day for a cow. This objective emphasizes the importance of fly control during the summer months. In order to maintain milk weight during the summer months, effective fly control measures should be implemented.

It was also determined that common *Staphylococcus* mastitis pathogens present in milk samples, can also be found in horn fly populations. *Staph. aureus*, *Staph. chromogenes*, and *Staph. agnetis* were all found to be present in both milk samples and horn fly pools, with the addition of *Staph. hyicus* present in horn fly pools. Horn flies carrying *Staph. chromongenes* had an association with a cow and herd testing positive for that same bacteria. No relationship was found for *Staph. aureus* or *Staph. agnetis*. Female horn flies are more likely to carry *Staph.*

aureus, *Staph. chromogenes*, and *Staph. agnetis* and are more likely to be caught off the ventral midline area than male horn flies. The results from this study suggests controlling female horn flies, specifically found on the ventral midline, could reduce the transmission of *Staphylococcus* mastitis pathogens on farm. Further research is needed to determine if the horn fly pools are carrying the same *Staph.* strain as those found in the milk samples and to determine control measures for strictly controlling for female horn flies on the ventral midline.

Overall, our study determined that if the producers are controlling for horn flies on their operation, the reduction in milk weight during the summer months may not be as severe. It may also be suggested that producers should focus on controlling specifically for female horn flies found around the cow's ventral midline area, as female horn flies were more likely to carry *Staph. aureus*, *Staph. chromogenes*, and *Staph. agnetis* and to be collected off the ventral midline area. Further research is needed to determine control strategies specifically for female horn flies found on the ventral midline. Dairy producers should consider some form of fly management on farm during the summer months.

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Appendix

Table 2.1: Mean SCC (± SD) for the 15 focal cows[1] and all cows[2] from each farm throughout the study (cells/mL)

Month	Farm 1	Farm 2	Farm 3	Farm 4
May	42,813 (± 56,188)[1]	--	--	145,533 (± 198,550)
	284,160 (± 620,978)[2]			133,108 (± 157,700)
June	82,470 (± 127,378)	61,310 (± 45,941)	69,594 (± 46,504)	80,133 (± 53,520)
	265,529 (± 576,902)	124,647 (± 111,847)	199,661 (± 360,456)	90,527 (± 78,687)
July	486,061 (± 1,601,671)	172,608 (± 273,806)	53,000 (± 107,423)	92,785 (± 100,300)
	470,717 (± 1,188,426)	271,914 (± 389,133)	217,591 (± 344,096)	134,111 (± 201,941)
August	103,061 (± 258,258)	456,416 (± 826,084)	179,308 (± 418,472)	175,667 (± 173,034)
	263,312 (± 510,340)	382,451 (± 536,851)	273,492 (± 737,959)	158,863 (± 157,376)
Sept.	296,166 (± 915,168)	112,166 (± 130,200)	219,769 (± 330,489)	--
	236,006 (± 628,465)	188,400 (± 207,602)	272,800 (± 641,226)	
Oct.	184,710 (± 373,297)	112,528 (± 68,237)	137,090 (± 213,407)	--
	393,997 (± 770,039)	216,086 (± 351,688)	212,619 (± 624,171)	

-- Farm not collected during that month, making data unavailable

Table 2.2: Mean milk yield (± SD) for the 15 focal cows[1] and all cows[2] from each farm throughout the study (kg)

Month	Farm 1	Farm 2	Farm 3	Farm 4
May	23.5 (± 1.6) [1]	--	--	10.3 (± 3.0)
	23.1 (± 4.2) [2]			9.6 (± 3.0)
June	22.8 (± 3.9)	19.6 (± 3.6)	12.4 (± 3.0)	12.5 (± 2.8)
	23.1 (± 4.5)	18.6 (± 4.1)	12.2 (± 3.2)	10.3 (± 2.9)
July	22.3 (± 2.9)	10.3 (± 5.6)	13.2 (± 2.2)	18.0 (± 3.7)
	20.4 (± 3.2)	8.2 (± 4.9)	11.5 (± 3.3)	16.5 (± 3.4)
August	21.4 (± 2.6)	6.2 (± 2.1)	8.3 (± 1.9)	10.0 (± 2.8)
	20.1 (± 3.5)	6.2 (± 2.0)	7.3 (± 2.3)	9.6 (± 2.6)
Sept.	20.2 (± 2.1)	5.4 (± 2.1)	4.9 (± 1.2)	--
	19.5 (± 3.6)	6.1 (± 1.8)	5.0 (± 1.2)	
Oct.	16.7 (± 3.0)	9.7 (± 2.7)	6.9 (± 1.3)	--
	17.3 (± 4.7)	12.6 (± 3.7)	6.3 (± 1.6)	

-- Farm not collected during that month, making data unavailable

Table 2.3: Mean DIM (± SD) for the entire lactating herd for each farm throughout the study

Month	Farm 1	Farm 2	Farm 3	Farm 4
May	197 (± 118)	--	--	178 (± 44)
June	207 (± 101)	108 (± 33)	89 (± 23)	187 (± 73)
July	208 (± 48)	147 (± 35)	128 (± 17)	207 (± 65)
August	232 (± 47)	173 (± 36)	154 (± 18)	222 (± 97)
Sept.	263 (± 46)	202 (± 42)	189 (± 20)	--
Oct.	286 (± 51)	222 (± 51)	210 (± 12)	--

-- Farm not collected during that month, making data unavailable

Table 2.4: Fixed effects included in the multivariate model for logSCC and milk weight at the cow level and the associated P-value, estimate and standard error

Fixed Effect	logSCC			Milk Yield		
	P value	**Estimate**	**Standard Error**	**P value**	**Estimate**	**Standard Error**
Total number of horn flies per cow	--	--	--	0.003	-0.01	0.003
DIM	0.003	0.003	0.0009	0.02	-0.02	0.008
Parity	0.3	0.04	0.04	0.2	-0.4	0.4
THI	--	--	--	--	--	--
Season	0.1	-0.1	0.08	0.04	-1.4	0.7
logSCC	--	--	--	0.008	-1.3	0.5
Milk weight	0.006	-0.02	0.007	--	--	--

--Not included in final model

Table 2.5: Fixed effects included in the multivariate model for logSCC and milk weight at the herd level and the associated P-value, estimate and standard error

Fixed Effect	logSCC			Milk Yield		
	P value	Estimate	Standard Error	P value	Estimate	Standard Error
Total number of horn flies per cow	0.14	-0.0008	0.0005	0.03	-0.03	0.01
DIM	--	--	--	0.2	-0.03	0.02
Parity	--	--	--	--	--	--
Season	0.2	0.06	0.05	--	--	--
logSCC	--	--	--	0.05	-10.9	5.2
Milk weight	0.1	-0.01	0.007	--	--	--

--Not included in final model

Table 3.1: Primers used for the multiplex PCR to identify to the species level and differentiate between Staph. species (Shome et al., 2011, Adkins et al., 2017)

Gene	Primer	Sequence (5' to 3')	Amplicon size (bp)	Positive Species
aroD	aroD-HyF	TAT GGT GTC GAC CAA TCG AAG GCT	425	*Staph. hyicus*
aroD	aroD-HyR	ACC CTA TAG CCC GCT TAC TT		
aroD	aroD-AgF	CGC ATG AGA GAC CAA TAC GCT	293	*Staph. agnetis*
aroD	aroD-AgR	TAG GAC GTA TAG AGG TGG		
23S	SAS2F	AGC GAG TCT GAA TAG GGC GTTT	894	*Staph. aureus*
23S	SAS2R	CCC ATC ACA GCT CAG CCT TAA C		
sodA	SCHS1F	GCG TAC CAG AAG ATA AAC AAA CTC	222	*Staph. chromogenes*
sodA	SCHS1R	CAT TAT TTA CAA CGA GCC ATG C		

Table 3.2: Fixed effects within the focal cow population associated with a cow milk sample testing positive for Staph. aureus, Staph. chromogenes, or Staph. agnetis

	Staph. aureus		***Staph. chromogenes***		***Staph. agnetis***	
	P-value	**Estimate**	**P-value**	**Estimate**	**P-value**	**Estimate**
THI	0.3	0.003	0.1	0.004	--	--
Fly Positive	0.2	-0.08	0.02	0.1	0.6	-0.02
Fly positive*total flies	0.2	0.0007	0.2	-0.0004	0.7	0.0001
Parity	0.4	-0.02	--	--	0.3	-0.02
Total number of flies per cow	0.3	-0.0005	0.4	0.0002	0.6	-0.0002

Table 3.3: Fixed effects associated with herd-level risk factors for milk samples testing positive for *Staph. aureus*, *Staph. chromogenes*, or *Staph. agnetis*

	Staph. aureus		***Staph. chromogenes***		***Staph. agnetis***	
	P-value	**Estimate**	**P-value**	**Estimate**	**P-value**	**Estimate**
Average fly positives	0.4	0.09	0.02	0.2	0.6	0.01
Average total number of flies per cow	0.3	0.0004	0.3	0.0003	0.9	-0.00002

Table 3.4: Probability of a horn fly pool to carry *Staph. aureus*, *Staph. chromogenes*, *Staph. agnetis*, and *Staph. hyicus* depending on location of collection

Organism	Location	Location	Estimate	Standard Error	Pr > |t|	OR	95% CI
Staph. aureus	Dorsal Midline	Ventral Midline	-0.6	0.2	0.001	0.5	0.4, 0.8
Staph. chromogenes	Dorsal Midline	Ventral Midline	-0.4	0.2	0.07	0.7	0.5, 1.0
Staph. agnetis	Dorsal Midline	Ventral Midline	-0.6	0.2	0.002	0.5	0.4, 0.8
Staph. hyicus	Dorsal Midline	Ventral Midline	-0.005	0.3	0.9	0.9	0.5, 1.9

Table 3.5: Probability of male and female horn fly pools to carry *Staph.* organisms

Organism	Sex	Sex	Estimate	Standard Error	Pr > |t|	OR	95% CI
Staph. aureus	Female	Male	0.6	0.2	0.003	1.8	1.2, 2.6
Staph. chromogenes	Female	Male	0.5	0.2	0.02	1.6	1.0, 2.4
Staph. agnetis	Female	Male	1.3	0.2	< 0.0001	3.5	2.3, 5.3
Staph. hyicus	Female	Male	-2.4	0.4	< 0.0001	0.09	0.04, 0.2

Table 3.6: Fixed effects associated with a fly pool testing positive for *Staph. aureus*, *Staph chromogenes*, *Staph. hyicus*, and *Staph. agentis*

	Staph. aureus		***Staph. chromogenes***		***Staph. agnetis***		***Staph. hyicus***	
	P-value	**Estimate**	**P-value**	**Estimate**	**P-value**	**Estimate**	**P-value**	**Estimate**
Location of Collection	0.0008	-0.2	0.06	-0.08	0.002	-0.1	0.9	-0.001
Sex	0.003	0.1	0.02	0.1	< 0.0001	0.3	< 0.0001	-0.2
Total number of flies per cow	--	--	0.05	-0.0004	--	--	--	--

Table 3.7: Bacteria previously identified using the API Staph System were reevaluated by enriching the original milk sample and then confirmed by PCR

	PCR Result								
API Result	None	*Staph. aureus*	*Staph. chromogenes*	*Staph. agnetis*	*Staph. aureus* + *Staph. chromogenes*	*Staph. aureus* + *Staph. agnetis*	*Staph. chromogenes* + *Staph. agnetis*	*Staph. aureus* + *Staph. chromogens* + *Staph. agnetis*	Total
Staph. aureus		x x x x			x x x x x			x	10
Staph. chromogenes	x x x		x x x	x x	x x x x x x x x x x x x x x x x	x x x x	x	x x	33
Staph. agnetis									0
Staph. hyicus	x x x		x	x	x x x x x		x		11
Total	6	4	4	3	28	4	2	3	

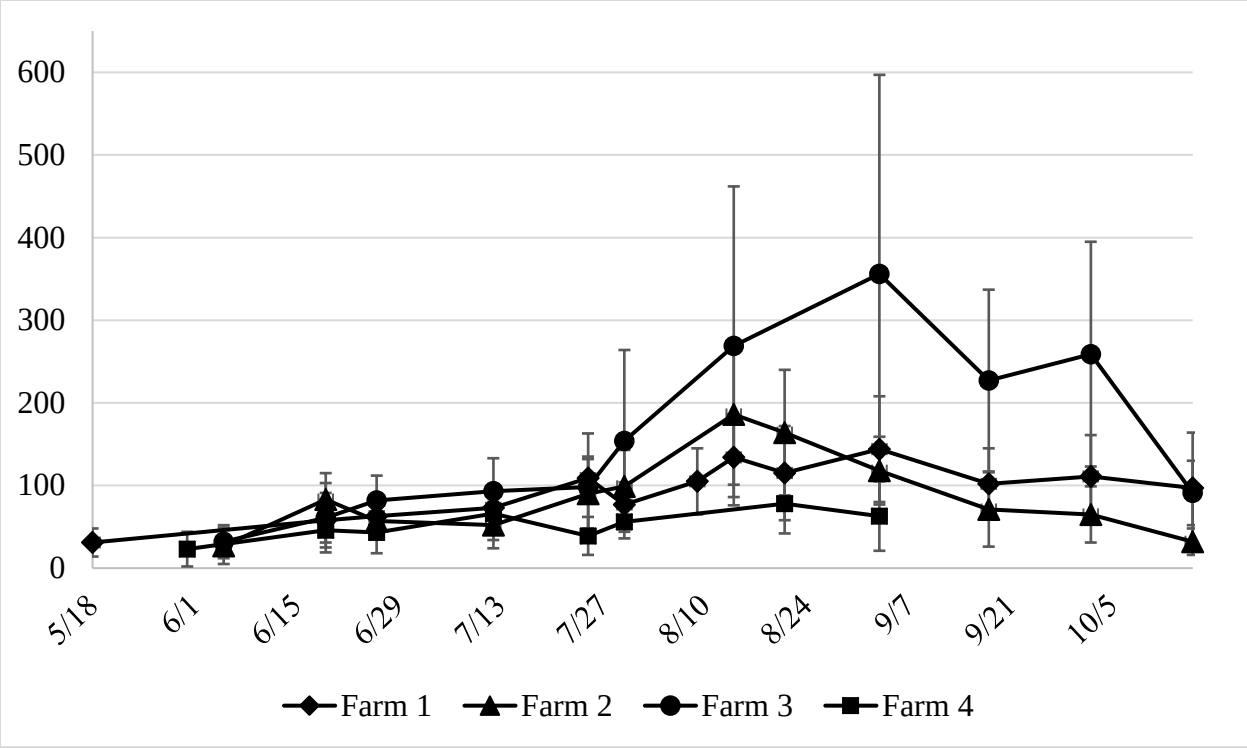

Figure 2.1: The mean number of horn flies on 15 focal cows per farm for each collection date. Error bars indicate (± SD). Mean number of horn flies increase throughout the summer, but varied by farm.

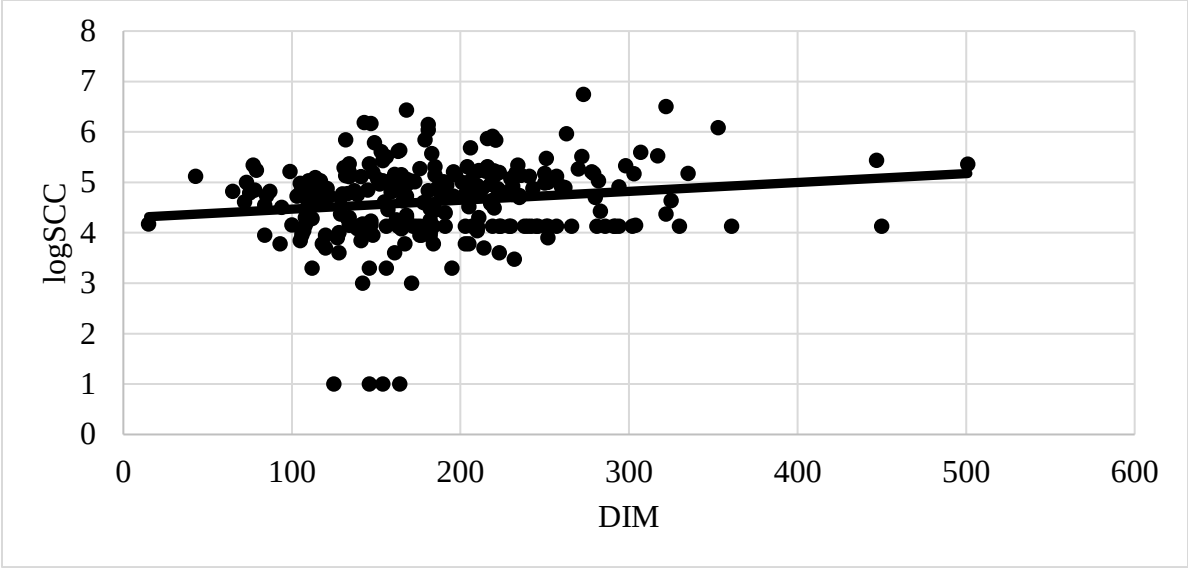

Figure 2.2: The variation in logSCC with DIM. Each dot represents one of the 15 focal cows on a given sample date. When testing the effects of mean horn fly numbers (P = 0.4), DIM (P = 0.003), parity (P = 0.3), milk weight (P = 0.006), and season (P = 0.1) on logSCC, there was a significant effect of DIM.

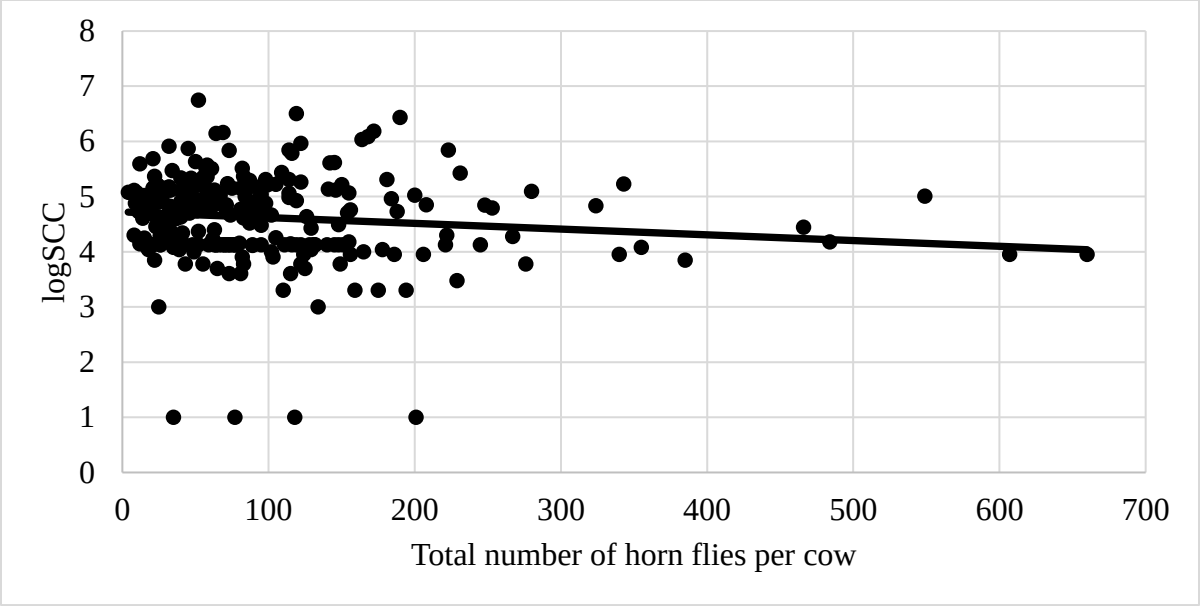

Figure 2.3: The variation in logSCC with total number of horn flies per cow. Each dot represents one of the 15 focal cows on a given sample date. When testing the effects of mean horn fly numbers ($P = 0.4$), DIM ($P = 0.003$), parity ($P = 0.3$), milk weight ($P = 0.006$), and season ($P = 0.1$) on logSCC, no relationship was found between logSCC and horn flies.

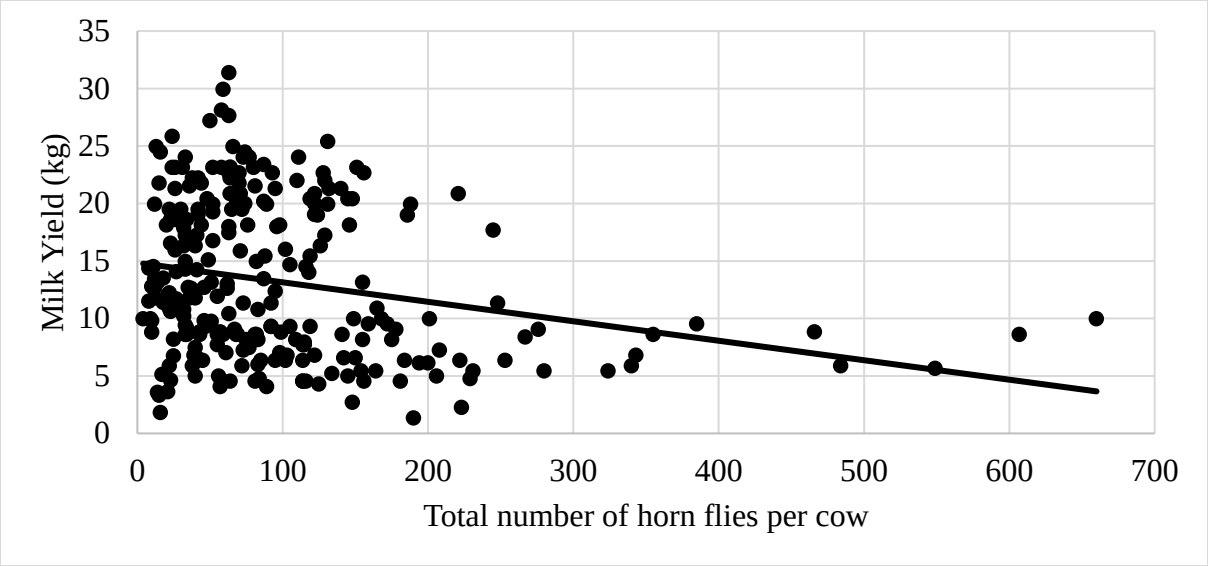

Figure 2.4: The variation in milk weight (kg) with total number of horn flies per cow. Each dot represents one of the 15 focal cows on a given sample date. When testing the effects of mean horn fly numbers ($P = 0.003$), DIM ($P = 0.02$), parity ($P = 0.2$), logSCC ($P = 0.008$), and season ($P = 0.04$) on milk yield, there was a significant negative effect of mean horn flies.

Vita

Emily was born and raised in Fremont, Ohio. She graduated high school from St. Joseph Central Catholic in 2014. She then received her Bachelor's of Science degree in Animal Science from The Ohio State University. During this time was when her passion for the dairy industry began. She worked at the ATI Dairy Facility in Wooster, Ohio for one summer and knew she wanted to help dairy farmers by providing them valuable research. In August 2018, she began her Master's of Science in Animal Science, where she was able to pursue her dream of helping dairy farmers and conduct applied research. After completing her degree, Emily plans to begin working within the dairy industry.