Intramammary infection in rapidly growing, non-lactating mammary glands

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ABSTRACT

Intramammary infections (**IMI**) are common in non-lactating heifer and dry cow mammary glands and occur during periods of appreciable mammary growth and development. The presence of these infections is expected to negatively impact mammary growth and development but has yet to be investigated. The works reported here investigated how IMI affects mammary tissue structure, cellularity, and the expression of integral mammogenic hormone receptors implicated in mammary growth. Non-pregnant non-lactating cows (n = 19) were administered estradiol and progesterone to stimulate mammary growth and 2 quarters of each cow were subsequently infused with either saline (n = 19) or *Staphylococcus aureus* (n = 19). Intramammary infusion of *Staphylococcus aureus* increased the number of immune cells present in gland secretions and also increased the proportion of neutrophils comprising these secretion somatic cells. Mammary tissues from quarters infused with *Staphylococcus aureus* contained more immune cells, less mammary epithelial tissue area, and greater tissue areas of intralobular stromal tissue than saline quarters. *Staphylococcus aureus* quarters also contained more apoptotic mammary epithelial cells and a lower proportion of apoptotic cells in the intralobular stroma compartment than saline infused quarters; this signified that *Staphylococcus aureus* quarters had less epithelial growth and experienced an expansion and/or lack of regression of stromal tissues. The number of cells expressing estrogen receptor α (**ESR1**) and progesterone receptor (**PGR**), as well as staining characteristics of ESR1 and PGR positive nuclei was also examined in these tissues. No appreciable differences were observed in any of the examined ESR1 and PGR measures between *Staphylococcus aureus* and saline mammary glands, but myoepithelial cells from *Staphylococcus aureus* glands had a greater nuclear staining area than saline quarters, indicating that these cells were affected by IMI. The results of these investigations indicate that IMI, in mammary glands that are concurrently stimulated to grow and develop, limits the growth of mammary epithelium and impairs regression of the stromal tissue, both of which are necessary for successful lactational performance.

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GENERAL AUDIENCE ABSTRACT

Successful growth and development of the dairy cow udder (mammary gland) is important and has long-term impacts on milk production. Most mammary growth occurs during the first pregnancy but, at this same time, a bacterial infection can be present within the mammary gland and is expected to hinder normal growth and development. The studies conducted here sought to examine how a bacterial infection, within a cow's udder, affects mammary gland growth and development. Overall, it was observed that a bacterial infection in the mammary gland reduced the amount of functional tissue that would eventually produce milk while simultaneously increasing the amount of connective tissue. Infected mammary glands also had a greater number of dying mammary cells, reducing the number of cells that would eventually produce milk. Estrogen and progesterone are known to be integral in supporting mammary growth, so an examination of the number of cells being able to receive signals from estrogen and progesterone was also undertaken; presence of an infection did not alter the number of cells able to receive estrogen and progesterone's signal. This work furthered our understanding of how bacterial infections affect mammary tissue and alter normal developmental processes.

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1.1. Bovine mastitis

Mastitis is defined as inflammation of the mammary gland and is a significant challenge for the Virginia, United States, and global dairy industries. Mastitis is the most common disease in the dairy industry (Barkema et al., 2009) and is almost exclusively the result of pathogenic bacteria entering the mammary gland via the teat canal, proliferating, and establishing an intramammary infection (**IMI**; Akers and Nickerson, 2011). Because mastitis is almost exclusively the sole result of an IMI, the two terms are frequently used interchangeably in the dairy industry. The economic losses from mastitis are profound and arise from: 1) decreased milk production and milk quality; 2) increased labor, veterinary costs, and drug usage; 3) discarding abnormal milk and milk containing antibiotics; and 4) prematurely culling of affected animals. In combination, these losses make mastitis the most expensive disease in the dairy industry (Barkema et al., 2009) with the greatest financial loss due to reduced milk production (Blosser, 1979; Safi et al., 2009).

During an IMI, neutrophils, macrophages, and lymphocytes are recruited to the mammary gland and migrate from the blood into mammary tissue and then the lumen of the gland to address invading pathogens (Harmon, 1994). This influx of immune cells raises the milk somatic cell count (**SCC**) which is comprised of epithelial cells, neutrophils, macrophages, and lymphocytes; the amount and proportions of these cells in milk varies whether the gland is healthy or experiencing an IMI. Indeed, infected glands have a greater proportion of immune cells comprising the SCC relative to healthy glands. This rise in the milk SCC is largely attributed to the increased number and proportion of neutrophils present in the milk (Paape et al., 2003; Akers and Nickerson, 2011). Healthy lactating cows typically have an SCC below 200,000 cells/mL of milk (Harmon, 1994; Schepers et al., 1997; Akers and Nickerson, 2011), with some having fewer than 50,000 cells/mL (Akers, 2002). On the contrary, a SCC above 200,000 cells/mL is indicative that the gland is likely infected (Schepers et al., 1997). In summary, milk SCC serves as a reliable measure of mammary inflammation and a predictor of IMI (Pyörälä, 2003), and its increase is negatively correlated with milk yield (Jones et al., 1984).

1.2. Classification of intramammary infection

Mastitis events and IMI differ considerably in duration, severity, and signs presented. Although many different classifications exist for describing a mastitis event or an IMI (Claxton and

Ryan, 1993), mastitis can be broadly described as being either clinical or subclinical (Vanderhaeghen et al., 2014). Clinical mastitic events display signs that are grossly observable, such as glands that are hot to the touch and exhibit localized inflammation and redness; milk from affected glands is also visually abnormal, exhibiting clots, flakes and/or blood. Subclinical mastitis is not accompanied with visual signs; therefore, a diagnostic test is required to detect this form of the disease such as measuring SCC, evaluating milk composition, and culturing milk to identify known bovine pathogenic bacteria to diagnose the presence of an IMI. Although the impacts of mastitis are most striking in clinically infected glands, subclinical IMI are 15 - 40 times more prevalent (Akers, 2002) and account for the largest monetary loss to the producer. These losses are in the form of decreased milk yield, decreased milk quality, and the loss of bonus premiums that processers may pay for high quality milk.

1.3. Mastitis pathogens

A multitude of different pathogenic bacteria, residing in an array of different reservoirs can infiltrate the mammary gland and establish an IMI. Given the abundance of different bacteria species and reservoirs, mastitis is regarded as a multifactorial disease which makes controlling IMI incidence difficult. Mastitis pathogens are grossly characterized based upon their reservoir, or where the pathogens reside; this reservoir is related to the pathogen's mode of transfer to the healthy mammary gland. For example, mastitis pathogens are classified as being either contagious, environmental, or opportunistic pathogens. The principal reservoir of the contagious pathogens is the infected mammary gland with transmission usually occurring during routine milking from infected to non-infected glands. The environmental and opportunistic pathogens are classically non-contagious by nature, and their main reservoirs are the environment of the dairy animal and the teat skin of the gland, respectively.

1.3.1. Contagious pathogens

Staphylococcus aureus, *Streptococcus agalactiae*, and the *Mycoplasma* spp. are the key contagious bovine mastitis pathogens and are mainly controlled through hygienic milking practices and postmilking teat disinfectants (Neave et al., 1966; Pankey et al., 1984). *Staph. aureus* is the most prevalent contagious mastitis pathogen in the United States with 43% of bulk tank milk samples testing positive during a national survey (USDA-APHIS, 2008). *Staph. aureus* infections in lactating cows range in severity in which severe clinical infections might result in the loss of

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functional quarters or more rarely, death (Fox and Gay, 1993), to subclinical infections that may clear with antibiotic treatment, albeit success rates are low (Barkema et al., 2006). Microabscesses can form as a result of *Staph. aureus* infection making antibiotic treatment less effective (USDA-APHIS, 2008). Because the formation of microabscesses and reduced response to antibiotic therapy, many *Staph. aureus* infections become chronic and persist throughout lactation with intermittent episodes of clinical mastitis (Fox and Gay, 1993; USDA-APHIS, 2008).

The prevalence of *Strep. agalactiae* on dairies is much less than that of *Staph. aureus* in the United States, with only 2.6% of dairies testing positive for the pathogen in bulk tank milk samples (USDA-APHIS, 2008). Few *Strep. agalactiae* infections in lactating cows display clinical signs; however, infections can contribute to large increases in bulk tank SCC and extensive milk secretory tissue damage can result from undetected and untreated infections (Akers, 2002). *Strep. agalactiae* infections are sensitive to the effects of appropriate antibiotic treatment, hence the reason for low prevalence on US dairies.

Mycoplasma spp. prevalence is also relatively low on US dairies with only 3.2% of US dairies generating positive bulk tank milk samples (USDA-APHIS, 2008). Despite this low prevalence, *Mycoplasma* spp. pose a considerable threat to the dairy industry. Its prevalence on dairies is positively associated with herd size, a phenomenon that is unique to this contagious pathogen (USDA-APHIS, 2008), and infections can result in substantial milk loss even though most infections are mild or subclinical (Hale et al., 1962; Fox, 2012). Likewise, mycoplasma infections do not respond to antibiotic treatment, especially β-lactam antibiotics due to the bacterium's lack of a cell wall. Consequently, it is often advocated to cull infected animals from the herd to control the spread of this pathogen. Although *Mycoplasma* spp. are considered a contagious pathogen that spreads during routine milking, it also colonizes other body sites and can spread hematogenously through blood and lymph (Jain et al., 1969). These additional reservoirs, and the fact that *Mycoplasma* spp. can spread hematogenously, support the hypothesis that IMI associated with *Mycoplasma* spp. is not solely the result of udder-to-udder transmission as discussed by Punyapornwithaya et al. (2011), Fox (2012), and Enger et al. (2015).

1.3.2. Environmental pathogens

Streptococcus uberis, *Streptococcus dysgalactiae*, *Escherichia coli*, and *Klebsiella* spp. are the classical environmental mastitis pathogens. In contrast to the contagious pathogens whose

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transmission and incidence is controlled by hygienic milking practices, environmental IMI incidence is largely controlled by reducing pathogen load in the environment (Hogan and Smith, 2012) and premilking teat disinfectants (Pankey and Drechsler, 1993). Approximately half of *Strep. uberis* and *Strep. dysgalactiae* IMI produce clinical signs (Todhunter et al., 1995). Antibiotic treatment of the environmental streptococci, *Strep. uberis* and *Strep. dysgalactiae*, infections is warranted during lactation in clinical cases (Hillerton and Berry, 2003). But, most often dry cow therapy, treating non-lactating cows at the end of lactation with an effective antibiotic in combination with a teat sealant, is effective in preventing and clearing environmental streptococci IMI (Godden, et al., 2003).

Escherichia coli and the *Klebsiella* spp. are coliform bacteria and are widespread in the dairy cow's environment. Approximately 85% of coliform IMI present clinical signs of infection (Hogan and Smith, 2012). Coliform infections can account for a large portion of the total number of clinical cases of mastitis on the farm despite the fact that prevalence in most herds is less than 5% (Hogan and Smith, 2012). Coliform infections are typically not treated with antibiotics as efficacy is limited and infections typically clear without intervention. To reduce the incidence of coliform IMI, it is advocated that a teat sealant be used in combination with dry cow therapy based on the findings of Huxley et al. (2002).

1.3.3. Opportunistic pathogens

The coagulase-negative staphylococci (**CNS**) comprise the greater proportion of the opportunistic pathogens and postmilking teat disinfectants are efficacious in reducing the incidence of CNS IMI (Quirk et al., 2012). In lactating cattle, the CNS are the most common pathogens associated with IMI (Piessens et al., 2011). Coagulase-negative staphylococcal IMI seldom present clinical signs, yet infections may persist throughout lactation while other are short lived and spontaneously cure. Infections associated with CNS typically do not receive antibiotic therapy as the majority fail to present clinical signs which is why infections may not always be detected.

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CHAPTER 2: Impact of intramammary infection on bovine mammary growth and development

2.1. Introduction

Without a doubt, the majority of mastitis research in the dairy industry has focused on lactating animals. As a result, profound improvements have been made in controlling the incidence of new intramammary infection (**IMI**) in lactating animals. Regardless of this achievement in lactating animals, non-lactating dairy animals such as cows that have completed a lactation or heifers that have yet to enter lactation can also experience IMI.

Once the dairy cow completes a lactation and enters the dry period, the mammary gland will involute and regress to a quiescent state due to milking cessation and milk stasis. The dairy cow is at an elevated risk for developing an IMI at the beginning of the dry period (Eberhart, 1986). The reasons for this increased IMI risk are numerous. First, bacteria that have entered the streak canal will no longer be removed or flushed from the streak canal during routine milking, which can potentiate a pathogen's opportunity to migrate up into the mammary gland. Additionally, with the cessation of daily milking, milk will begin to accumulate within the udder and teats may become "leaky". Milk leaking from the teat end increases a quarter's risk of developing an IMI during the dry period (Klaas et al., 2005). In addition, any milk that has leaked from the teat end can remain on the teat skin and create an ideal environment for bacterial growth. This increased pathogen load on the teat skin would increase the new IMI risk (Pankey et al., 1984). The termination of daily cleaning and dipping of teats in a disinfectant during milking is expected to also influence the number of bacteria that would be present on the teat end, and ultimately influence new IMI risk. In summary, the dry period is a time when a dairy cow experiences a significant increase in her risk to develop a new IMI; to combat this problem, many commercial products and management practices have been developed and applied to reduce the incidence of IMI in dry dairy cows.

Aside from cows being susceptible to IMI during lactation and the dry period, nulliparous heifers also experience IMI. Currently, a limited number of studies have investigated the consequences of IMI in nulliparous heifers in detail despite its documented occurrence in the dairy industry over 70 years ago (Schalm, 1942). The impacts of IMI on milk yield and mammary biology are well understood in lactating cattle (Akers and Nickerson, 2011); on the contrary, the consequences of IMI in non-lactating glands of nulliparous heifers are less well defined. The

purpose of this review and the reports described herein is to better define the occurrence of IMI in nulliparous heifers and how such IMI are expected to impact mammary growth and development.

2.2. Intramammary infection in nulliparous heifers

Intramammary infections in nulliparous heifers occur in both non-pregnant and pregnant animals and can be identified as early as 6 months of age (Nickerson, 2009). Although IMI can be present at this extremely early age, most investigative reports have described new IMI in heifers at breeding age, during first gestation, and around the periparturient period (Boddie et al., 1987; Trinidad et al., 1990b; Pankey et al., 1991; Aarestrup and Jensen, 1997; Owens et al., 2001; Oliver et al., 2003; Oliver et al., 2004). This risk of IMI increases as gestation progresses (Trinidad et al., 1990b; Fox et al., 1995). Trinidad et al. (1990b) surveyed primigravid heifers in four Louisiana herds and reported that 60.8% of all quarters were infected during first trimester, and an overwhelming 76.6% of quarters were infected in heifers during their third trimester of gestation. Fox et al. (1995) reported similar patterns when researchers examined 28 herds across the United States during the spring, summer, fall, and winter seasons. Overall, 29% of the quarters sampled during the first trimester of gestation were identified as being infected, whereas 44.6% of quarters sampled during the third trimester were infected. Within this report, Louisiana had the highest prevalence of both *Staphylococcus aureus* and CNS IMI compared to the herds surveyed in Vermont, California, and Washington State. It is obvious that herd location significantly influences IMI risk, and these differences are likely attributed to differences in environmental factors and management practices. Nonetheless, it can be concluded that primigravid heifers are at greatest risk of having an IMI during their last trimester of gestation.

Most heifers with an IMI do not display visible signs of mastitis. In the survey by Trinidad et al. (1990b), only 15.1% of the infected quarters presented clinical signs of infection. Additional studies examining IMI in primigravid heifers on commercial dairies have reported that only 7.5% of infected quarters present clinical sings of mastitis (Nickerson et al., 1995). This lack of visual detection likely has contributed to why little research has focused on IMI in nulliparous heifers since the majority of infections are not detected and thus not identified as a problem.

2.3. Mastitis pathogens associated with nulliparous heifer intramammary infection

The lack of clinical signs in nulliparous IMI is likely a function of both the causative pathogens and the physiological state of the gland itself. During first pregnancy, the gland is in a

semi-quiescent state, with mainly mammogenesis, colostrogenesis, and to a lesser extent, lactogenesis occurring. Because the gland is not synthesizing and secreting copious amounts of milk and also subject to a different environment, the profile of mastitis pathogens associated with IMI in nulliparous heifers differs slightly from that of lactating glands. It is suspected that this is a result of the differences in nutrients for the bacteria to grow, and differences in management between lactating cows and nulliparous heifers (i.e. exposure to mastitis pathogens in the milking parlor). Investigative survey studies have been conducted and were recently reviewed (Fox, 2009; De Vliegher et al., 2012) to better define the causative agents responsible for IMI in primigravid heifers, which are listed below.

2.3.1. Coagulase-negative staphylococci

The coagulase-negative staphylococci (**CNS**) are the most common mastitis pathogens recovered from primigravid heifer mammary glands, as an average of 28.6% of all quarters are diagnosed as infected prior to first calving (Fox, 2009). To date, over 20 different CNS spp. have been isolated from the bovine mammary gland (Vanderhaeghen et al., 2014). Despite the abundance of the many different CNS spp., the prevalence of these species differ and this is partiality due to differences in geographic location (Supre et al., 2011; Quirk et al., 2012). Additionally, some species appear to have a greater affinity for affecting mammary health more than others (Supre et al., 2011). *Staphylococcus chromogenes* is the predominant CNS spp. isolated from both milk (Vanderhaeghen et al., 2014) and nulliparous heifer mammary glands (Boddie et al., 1987; Trinidad et al., 1990b; Aarestrup and Jensen, 1997). *Staphylococcus xylosus*, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Staphylococcus simulans*, (Vanderhaeghen et al., 2014) and *Staphylococcus hyicus* (Harmon and Langlois, 1989) are also highly prevalent and commonly isolated from lactating and non-lactating bovine mammary glands.

Based on the earlier discussion, the CNS spp. contribute to the normal flora of the teat skin, allowing them "opportunity" to colonize the streak canal, migrate into the gland, and establish an IMI. However, the CNS spp. also colonize other body sites and are prevalent in the environment of the dairy cow. Reservoirs include, but are not limited to the nares, vagina, hair coat, stall air, stall floors, sawdust bedding, and human skin (White et al., 1989; Thorberg et al., 2006; Piessens et al., 2011; Piessens et al., 2012). This abundance of reservoirs makes understanding the etiology of the CNS IMI difficult as the reservoir appears to greatly influence what species might be present. For

example, *Staph. chromogenes* is infrequently found in the environmental reservoirs (Piessens et al., 2011) but appears to preferentially occupy the skin and teat canal (De Vliegher et al., 2003). Conversely, *Staphylococcus equorum* and *Staphylococcus fleurettii* reside almost exclusively within environmental reservoirs and are less commonly isolated from milk (Piessens et al., 2011). Furthermore, speciation of different CNS spp. has not been routinely employed in previous investigations, which has limited our ability to understand the etiology of each CNS spp.

2.3.2. *Staphylococcus aureus*

Staph. aureus is the second most prevalent mastitis pathogen in primigravid heifer glands. On average, 3.5% of all quarters are infected with *Staph. aureus* prior to calving (Fox, 2009) although quarter infections can range from 14.9% (Trinidad et al., 1990b) of quarters to only 0.4% prevalence (Aarestrup and Jensen, 1997). This range is attributed to differences in management practices and environmental factors. Roberson and colleagues (1994; 1998) investigated the possible reservoirs of *Staph. aureus* in dairy heifers and concluded that *Staph. aureus* is commonly found on mammary gland skin, muzzle, and lacteal secretions; these are important reservoir for this pathogen. Some of the same *Staph. aureus* strains that were isolated from these body sites were also identified in heifer colostrum at parturition and isolated from milk of lactating cattle (Roberson et al., 1998). *Staph. aureus* has also been isolated from other sources such as bedding, feedstuffs, insects, and dairy workers but, this isolation is of a lesser degree and inconsistent between herds (Roberson et al., 1994). The importance of each specific reservoir in heifer IMI is less clear and likely to be herd specific.

Staph. aureus is a classic contagious pathogen with transmission for lactating cows most commonly occurring in the milking parlor; all the while, it is present in the glands of nulliparous heifers that have never entered the milking parlor. Clearly, other transmission pathways of infection must exist. Owens et al. (1998) demonstrated that horn flies (*Haematobia irritans*) contribute to the transmission of *Staph. aureus* from infected to non-infected nulliparous heifer glands. In their study, researchers allowed naïve horn flies to become colonized with *Staph. aureus* via exposure to culture and then feed on the teats of non-infected heifers. This feeding on the teats resulted in scab formation and the colonization of these scabs with *Staph. aureus*. By the same token, naïve horn flies were allowed to feed on the infected heifers' teats and were consequently colonized by *Staph. aureus*. Moreover, Louisiana herds not practicing some type of fly control had a 5.8-fold greater

number of *Staph. aureus* IMI in nulliparous heifers compared to herds with some type of fly control (Nickerson et al., 1995). Horn flies clearly influence the prevalence of heifer *Staph. aureus* IMI.

2.3.3. Environmental pathogens

Collectively, the environmental pathogens infect approximately 6.8% of nulliparous heifer quarters based on the weighted average of studies summarized by Fox (2009). Although this percentage of infected quarters is obviously higher than that of the *Staph. aureus* infected quarters, it is important to recognize that many different species contribute to this percentage. Consequently, species specific prevalence in nulliparous heifer glands is less clear and can be expected to be less than *Staph. aureus* based on the reports of Boddie et al. (1987), Myllys (1995), and Oliver et al. (2004). Because the prevalence of individual environmental pathogen species is less clear and fluctuates by season, location, and herd, the significance of environmental pathogen in nulliparous heifer IMI is less well understood and will not be discussed further in this review.

2.4. Significance of nulliparous heifer intramammary infection

Heifers entering the milking herd are the future of the dairy farm. These replacement heifers represent a sizable investment to the producer and will impact the herd's future milk production and genetic progress. Problems with heifers entering the milking herd can have long term consequences on herd productivity and profitability.

Heifers infected with contagious mastitis pathogens during first gestation can serve as an infectious reservoir. Consequently, transmission of these contagious pathogens to herd mates through vectors (e.g. horn flies) can result in the infection of both non-lactating and lactating herd mates. Furthermore, infections in prepartum heifers commonly persist into lactation (Oliver et al., 1992; Myllys, 1995; Nickerson et al., 1995; Aarestrup and Jensen, 1997; Oliver et al., 2004; Borm et al., 2006). Heifers freshening with an IMI are expected to have elevated SCC during early lactation and are at greater risk to be prematurely culled compared to those herd mates not exhibiting an increased SCC during early lactation (De Vliegher et al., 2005). This increased culling risk is, in part, a consequence of the greater likelihood of future milk tests producing elevated SCC and the reduced milk yield that was associated with affected heifers (De Vliegher et al., 2005).

It is well established that heifers freshening with IMI produce less milk than healthy heifers. Oliver et al. (2003) demonstrated that simply treating heifers 14 days prepartum with a cephapirin antibiotic increased total milk yield by 10.2% over the first lactation compared to herd mates not

receiving a blitz prepartum antibiotic therapy. Owens et al. (1991) reported similar results when primigravid *Staph. aureus* challenged heifers received prepartum antibiotic therapy and produced 13.1% more milk on a daily basis than challenged heifers not receiving antibiotic therapy. Beyond the obvious milk yield losses that accompany heifers freshening with an IMI, an IMI during first gestation can be suspected of compromising mammary development. First pregnancy in the bovine is the time when the mammary gland experiences its initial development to prepare for lactation, and this first gestation accounts for the greatest amount of mammary growth (Swanson and Poffenbarger, 1979; Tucker, 1987). This initial development during first pregnancy serves as the foundation for the animal's productive life. Obviously, insults to the mammary gland during this most important growth period would conceivably have long lasting impacts on the animal's future productive life, adding to the unrealized costs of this disease.

2.5. Mammary tissue changes associated with intramammary infection

In the most general sense, alveolar/ductal lumen resident and tissue resident macrophages, as well as mammary epithelial cells, serve as sentinels for detecting the presence of pathogenic bacteria. When these cells become activated, via contact with infectious pathogens or their toxins, chemokines are released to recruit immune cells to the site of infection. In the instance of the lactating bovine, neutrophils are the primary immune cells recruited (Jain et al., 1969; Nickerson, 1980; Paape et al., 2003; Akers and Nickerson, 2011; Wellnitz and Bruckmaier, 2012). These neutrophils are found in the blood, where they roll along the apical surface of the endothelial cells waiting to transverse the endothelial barrier and enter the mammary tissue when recruited. The activated neutrophils, through diapedesis, would migrate through the tissue to enter the alveolar or ductal lumen. Although this immune response is necessary to clear the invading pathogen, it is not without consequence to the mammary gland.

The common histological and cytological changes associated with mastitis in lactating mammary glands are well understood and have been previously reported and reviewed by numerous authors (Spencer, 1949; Helmbolt et al., 1953; Chandler and Reid, 1973; Chandler et al., 1974; Akers and Nickerson, 2011). In the review by Akers and Nickerson (2011), the authors noted that mammary glands experiencing a mastitis event exhibited: 1) a decreased percentage of tissue area occupied by the alveolar lumen; 2) a decreased percentage of epithelium tissue area; 3) an increased percentage of mammary tissue stromal area; 4) shedding of secretory mammary

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epithelial cells into the milk; and 5) occlusion of ducts by fibrin contributing to milk stasis and loss of epithelial secretory potential. Akers and Nickerson (2011) also noted from the ultrastructural portion of their review that secretory epithelial cells appear to become dedifferentiated and adhere loosely to the basal lamina, resulting in epithelial cells being sloughed into the alveolar lumen. Secretory epithelial cells also became vacuolated and experienced varying degrees of degradation of organelles important for milk synthesis (mitochondria, Golgi complex, endoplasmic reticulum, etc.).

In a specific investigation, Akers and Thompson (1987) sought to characterize the effects of strictly inflammation and immune cell migration on mammary histological structures by infusing sterile oyster glycogen into healthy, lactating ovine glands to cause sterile mastitis. In this report, similar undesirable changes in histological structure were described illustrating the significant changes to mammary structures merely from leukocyte migration and accompanying inflammation. Moving away from the damage and histological changes elicited by an immune response, a report by Bayles et al. (1998) highlighted the damaging effects that mastitis pathogens alone, *Staph. aureus* in this instance, can have on the mammary epithelial cells unaccompanied by the invasion of immune cells. Researchers challenged confluent MAC-T cells with the *Staph. aureus* Novel strain (Smith et al., 1998) and observed apoptosis of the MAC-T cells. More recently, Hu et al. (2014) demonstrated that *Staph. aureus* challenge of primary mammary epithelial cells activated the FAS-FADD apoptosis pathway and increased activities of caspase 3 and 8. The effects of both inflammation and bacterial damage have been studied together with Long et al. (2001) demonstrating that intramammary infusion of *Escherichia coli* causes apoptosis of mammary epithelial cells in the lactating gland. In conclusion, both the pathogenic bacteria and immune cell migration and inflammation, in combination, and alone produce significant negative changes in mammary epithelial cells and mammary histological structure, and these changes are associated with reduced milk yield in lactating mammary glands.

2.5.1. Mammary tissue changes in nulliparous heifers experiencing intramammary infection

Fewer studies have examined and characterized the impacts that IMI has on mammary structure and function in non-lactating, heifer mammary glands. The study by Trinidad et al. (1990a) is perhaps the most comprehensive to date, and examined the effects that staphylococcal infections have on mammary tissue in non-pregnant, nulliparous heifers. In this investigation,

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researchers challenged unbred dairy heifers with *Staph. aureus* and collected tissues 2 – 3 weeks post-challenge for histopathologic examination and compared these tissues to those naturally infected with CNS and uninfected quarters. The percentages of epithelial and luminal areas in tissues collected from *Staph. aureus* infected quarters were less than those collected from uninfected and CNS infected quarters. Furthermore, a greater stromal area percentage was observed in *Staph. aureus* and CNS infected quarters relative to uninfected quarters, demonstrating that a higher amount of connective tissue was present in these affected quarters and a reduced amount of functional epithelium. The images below exemplify these marked histological differences observed between uninfected and *Staph. aureus* infected mammary tissues.

It is important to recognize that IMI can occur in heifers that are both non-pregnant and pregnant. Even though IMI occur in both physiological states, no reports could be identified that
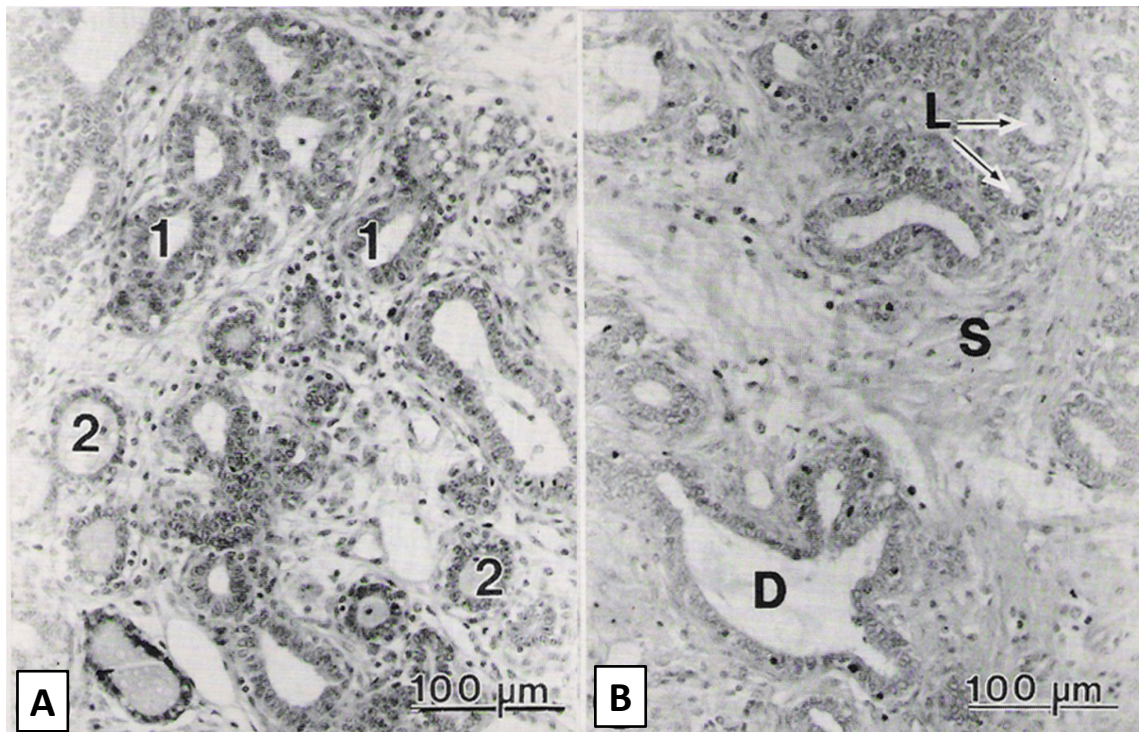
Figure 2.1. Non-pregnant heifer mammary tissues collected from uninfected (Panel A) and *Staph. aureus* infected quarters (Panel B). Numerous epithelial profiles (1), some containing mammary secretion (2), were observed in uninfected tissues. Conversely, fewer epithelial profiles were present in *Staph. aureus* infected tissues and a greater amount of stroma (S) was evident; luminal space (L) was also reduced. A large duct (D) is also shown. Images are from Trinidad et al. (1990a) and used with permission.

specifically investigate how IMI impacts pregnant heifer mammary glands that are experiencing rapid mammary growth and development to prepare for the onset of lactation. The absence of such reports in the literature is noteworthy.

2.6. Mammary growth and development during pregnancy

The bovine mammary gland is truly unique compared to most other organs given that the majority of mammary growth and development occurs postnatally. As stated earlier, the greatest amount of mammary growth and development occurs during first pregnancy (Tucker, 1987), in preparation for lactation. The number of individual studies quantifying and characterizing bovine mammary growth and development during first gestation is limited, which is likely a result of the logistics and expense that must be navigated to obtain mammary tissue needed for sampling during this period, e.g., total mammary tissue collections from pregnant animals.

Perhaps the most comprehensive characterization of bovine mammary growth and development occurring during first gestation is described in the report of Swanson and Poffenbarger (1979). In their study, 9 identical twin dairy heifer pairs were bred to initiate pregnancy associated mammary growth, and whole udders were harvested and examined at different stages of gestation to evaluate metrics of mammary growth and characterize changes in histological structure. From their gross evaluation of the excised glands, mammary parenchyma was observed to expand dorsally, from the teat, and replaced and/or displaced mammary fat pad tissues until approximately 8-9 months of gestation. This expansion coincided with a continued increase in udder mass as gestation progressed; mammary parenchymal mass also increased as gestation progressed. Mammary parenchymal DNA was also estimated in this report, and it was demonstrated that total mammary DNA increased as gestation progressed. Histologically, mammary tissues from a non-pregnant heifer contained ductal epithelial structures that were double layered, and the epithelial lumens were small and devoid of secretory products. This tissue appearance was relatively consistent until approximately the 5th month of gestation when both double and single layered epithelial structures could be observed; some secretory material could be observed in the epithelial lumens. The single layered epithelial structures were suspected to be rudimentary alveoli. During the 6th and 7th months of gestation, alveolar structures increased in size and number and more secretory products were present in epithelial lumens. At the 8th month of gestation, alveoli were larger still, and at 9 months of gestation, the tissue organization and

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appearance was similar to lactating mammary glands. A summary of the Swanson and Poffenbarger (1979) report is that bovine mammary growth occurred at an exponential rate as gestation progressed, increasing at a rate of 23.6%, for every consecutive month of pregnancy, and arrangement of tissues appeared to be most markedly altered during the last half of gestation when epithelial structures were arranged into alveoli.

Although the report of Swanson and Poffenbarger (1979) is likely the most comprehensive investigation of first gestation mammary growth to date, it is limited by low animal numbers and the fact that 3 different dairy breeds were utilized. The 3 different breeds of dairy animals would influence total mammary DNA, regardless of stage of gestation, and influence the estimated mammary growth patterns. Lastly, this work is significantly dated, 40 years previous, and considerable genetic selection and progress has unequivocally influenced the amount of secretory epithelium in the bovine udder. It is unknown how the growth and development metrics in this report correspond to today's animal, but it is logical to expect that the patterns of mammary development roughly parallel that of today's dairy animal.

2.6.1. Ovarian hormonal support of mammary growth

Because the mammary gland is an extension of the reproductive system, and its primary role is to support the nourishment and development of the neonate, it should not be surprising that its growth and development is largely directed by reproductive hormones. The importance of ovarian secretions in stimulating mammary growth and development has been recognized for decades but was first demonstrated in dairy heifers in the early 1950s. In a simple study, Wallace (1953) demonstrated that mere removal of ovaries in heifers, at birth, resulted in a complete failure of mammary gland parenchymal development relative to non-ovariectomized heifers when glands were examined at 6 months of age. However, when ovariectomized heifers were continuously administered stilboestrol, a nonsteroidal estrogen, rescue of mammary growth was demonstrated, underlining the importance of ovarian secretions, estradiol in this instance, for directing mammary gland growth. Today, numerous studies have built upon this work and explored how bovine mammary growth is influenced by the ovary and the classical ovarian steroids, estradiol and progesterone (Sud et al., 1968; Purup et al., 1993; Woodward et al., 1993; Berry et al., 2003; Meyer et al., 2006; Tucker et al., 2016; Geiger et al., 2017).

Estradiol is most classically recognized for stimulating proliferation of mammary epithelial cells (Woodward et al., 1993) and lengthening ductal structures, while progesterone is most implicated in promoting lobular alveolar formation, as discussed and reviewed by others previously (Cowie et al., 1980; Anderson et al., 1985; Shyamala, 1997; Connor et al., 2007). Connor et al. (2007) previously reviewed how these two pivotal hormones induce transcriptional changes in mammary epithelial cells and promote mammary gland growth and development. Most of estradiol's and progesterone's actions are achieved by these hormones binding to their respective nuclear receptors in mammary epithelial cells and inducing transcription of growth factors that direct mammary growth and morphogenesis.

In the instance of estrogen, 2 nuclear receptors have been described, but only estrogen receptor α (**ESR1**) has been described to be present in bovine mammary epithelial cells at levels detectable via immunohistochemistry (Connor et al., 2007). Upon estradiol binding to ESR1, this complex is thought to induce transcription by binding to hormonal response elements, or potentiate the transcription of growth factors by acting as a cofactor (as discussed by Connor et al. (2007)). Estradiol's effects in stimulating gland growth are believed to be a result of paracrine signaling from ESR1 positive nuclei in the epithelium and stroma (Connor et al., 2007). This mechanism is suspected because proliferating mammary epithelial cells are typically ESR1 negative (Clarke et al., 1997; Capuco et al., 2002).

In the instance of progesterone, two receptors have also been described, progesterone receptor (**PGR**) -A and PGR-B, but their precise roles in the mammary gland are less clear relative to estradiol, especially in the bovine. Connor et al. (2007) previously reported that PGR-A is approximately 3 times more abundant than PGR-B in the bovine mammary gland, but most previous investigations have not differentiated between the two isoforms (Schams et al., 2003; Tucker et al., 2016; Geiger et al., 2017). Like the effects of estradiol binding to ESR1, most of progesterone's effects are thought to affect neighboring cells via a paracrine mechanism of PGR positive cells producing factors that affect neighboring cells as proliferating mammary epithelial cells are also typically negative for PGR (Clarke et al., 1997).

Significant effort has been exerted to elucidating what factors are produced from ESR1 and PGR paracrine signaling, as well as to more completely understand these, and other factor's effects on gland growth and development. These factors and mechanisms have been discussed and

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reviewed by others previously (Hovey et al., 1999; Akers, 2006; Connor et al., 2007). A listing of the numerous factors implicated in directing mammary growth includes, but is not limited to, insulin-like growth factor (IGF), epidermal growth factor (EGF), hepatocyte growth factor, fibroblast growth factor, amphiregulin, and transforming growth factors α and β. Even though these factors are known to affect gland development, the interconnected web these factors play is immense, and is perhaps best described as the "growth factor soup" language used by Akers' (2006) review. Complicating the matter further is appreciating that the mammary gland's architecture is complex and comprised of many different cell types (various immune cells, fibroblasts, endothelial cells, adipocytes, mammary epithelial cells, and myoepithelial cells), which adds additional layers of complexity and difficulty in isolating the specific origins and actions of specific growth factors. This is why most investigations that evaluate the specific roles of these growth factors are *in vitro* rather than *in vivo*, especially when knockout models are not possible.

It is humbling to recognize and appreciate the complex orchestration of the different growth factors and cell types necessary for successful gland morphogenesis to occur. But aside from the specific growth factors that have been described to date, or will be described in the future, the pivotal roles of estradiol and progesterone acting as mammogenic hormones, simulating and directing mammary growth are clear.

2.6.2. Induction of mammary growth and lactation

Researchers have been interested in stimulating mammary growth and initiating lactation without the need for pregnancy for a considerable time. Evidence of this endeavor is widely abundant in the scientific literature and has been reviewed numerous times (Fulkerson, 1979; Cowie et al., 1980; Fulkerson, 1981; Kensinger and Magliaro-Macrina, 2011). In the bovine, variation in how to best achieve inductions of mammary growth and lactation is present, but most utilized protocols have centered around the use of estradiol and progesterone given their abundant documentation and involvement in mammary growth and lactogenesis. Almost all the historical induction protocols used in cattle before 1970 were long in duration (administration of hormones for greater than 1 month in duration), which made their utility, in a practical and research setting, limited.

In the early 1970s, Smith et al. (1971) sought to induce gross colostrogenesis and transport of IgG_1 into mammary gland lumens by injecting non-pregnant dry cows with supraphysiological

levels of estradiol and progesterone for 7 consecutive days. As a result, the udders of these experimental animals udders filled with a colostral-like fluid, which was examined for the specific objectives of the study. Smith and Schanbacher (1972) followed this work and applied this same induction protocol to non-pregnant, non-lactating dairy animals and successfully induced 7 of the 10 treated animals into lactation. In Smith and Schanbacher's discussion, they speculated that the successful induction of a virgin heifer into lactation was likely the result of the new formation of alveoli in this heifer's gland given the animal had not been gravid previously. This is speculated because the heifer's gland would have been inchoate and possessed limited parenchyma and no alveolar structures needed to synthesize and secrete milk. These researchers later examined nulligravid heifer's ability to produce milk in response to the protocol (Smith and Schanbacher, 1973) and reported similar success, indicating that this protocol supported the necessary growth of mammary tissues and formation of secretory structures. Given this protocol's success, it has been utilized and adapted many times over to investigate key mechanisms involved with mammogenesis, colostrogenesis, and lactogenesis (Smith et al., 1973; Narendran et al., 1974; Howe et al., 1975; Collier et al., 1976; Croom et al., 1976; Erb et al., 1976; Willett et al., 1976; Erb et al., 1977; Fulkerson, 1978; Ball et al., 2000; Macrina et al., 2011a; Macrina et al., 2011b; Mellado et al., 2011; Macrina et al., 2014; Stark et al., 2015).

2.7. Purpose of study and hypothesis

Although the disciplines of mastitis and mammary physiology are more often than not, separated, and independently investigated, it is clear that significant cross over exists, and a lack of understanding is present on how IMI affects mammary growth and gland morphogenesis. Based on previous works, it is expected that IMI negatively impacts mammary gland growth and morphogenesis during gestation and these impairments are expected to ultimately affect future milk yield; however, to date, no investigation has confirmed this. Given the chief roles of estradiol and progesterone in directing mammary gland growth and development, and their previous success in model settings to induce mammary growth and lactation, opportunity exists to stimulate mammary growth and concurrently challenge rapidly growing mammary glands with a mastitis pathogen. In doing so, an examination of the tissue, cellular, and molecular alterations arising from the IMI in these treated glands is possible. From a model standpoint, this approach allows for the clear examination of how IMI, and the associated immune response, affect mammary tissues

receiving signals to grow and develop. It is proposed to utilize a mammary growth induction model to stimulate rapid mammary growth and development, and challenge stimulated glands with a strain of *Staph. aureus* (*Staph. aureus* Novel; Smith et al. (1998) to allow for the elucidation of tissue morphology, cellular apoptosis and proliferation, and abundance of ESR1 and PGR changes in the experimental tissues.

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CHAPTER 3: *Staphylococcus aureus* intramammary challenge in non-lactating mammary glands stimulated to rapidly grow and develop with estrogen and progesterone

3.1. Abstract

Intramammary infections (**IMI**) are prevalent in non-lactating dairy cattle and their occurrence during periods of significant mammary growth and development (i.e. pregnant heifers and dry cows) is believed to interfere with growth, development, and subsequent milk production. However, direct study of IMI impacts on non-lactating but developing mammary glands is lacking. The objectives of this study were to 1) define how IMI affected total and differential mammary secretion somatic cell counts in mammary glands stimulated to rapidly grow using estradiol and progesterone, and 2) characterize changes in mammary morphology in response to IMI. Mammary growth was stimulated in 19 non-pregnant, non-lactating cows and 2 quarters of each cow were subsequently infused with either saline (n = 19) or *Staphylococcus aureus* (n = 19). Mammary secretions were collected daily until mammary tissues were collected at either 5 or 10 days post-challenge. *Staph. aureus* quarter secretions yielded greater concentrations of somatic cells than saline quarters and contained a greater proportion of neutrophils. *Staph. aureus* mammary tissues exhibited higher degrees of immune cell infiltration in luminal and intralobular stroma compartments than saline quarters. Infected tissues also contained reduced areas of epithelium and tended to have greater amounts of intralobular stroma. Results indicate that IMI in non-lactating glands that were stimulated to grow, resulted in immune cell infiltration into mammary tissues and secretions, which was associated with changes in mammary tissue structure. The observed reduction of mammary epithelium indicates that IMI impair mammary development in rapidly growing mammary glands, which may reduce future milk yields.

3.2. Introduction

Bovine mastitis is almost exclusively the result of a bacterial intramammary infection (**IMI**) and continues to be a major challenge for the US and global dairy industries. The effects of mastitis are most apparent, and appreciated, in lactating cattle given the considerable volume of literature that has described the increase in milk somatic cell count (**SCC**) (Dohoo and Leslie, 1991), reduced milk yields (Jones et al., 1984), and histopathological changes (Akers and Nickerson, 2011) that occur in response to IMI. Regardless of these well-documented effects in the lactating bovine mammary gland, it is recognized that both non-lactating heifers (Trinidad et al., 1990b; Fox et al., 1995) and dry cows (Eberhart, 1986) can also develop IMI.

Quarter IMI prevalence in nulliparous heifers is estimated to be approximately 43%, based on a weighted average of summarized survey studies (Fox, 2009) and approximately 8 – 25% of quarters in dry cows are expected to acquire new infections during the dry period between lactations, as summarized previously by Arruda et al. (2013). The occurrence of such IMI in non-lactating mammary glands is concerning given the considerable mammary growth that occurs during these distinct physiological states. For instance, the greatest amount of mammary growth and development that transpires during an animal's life occurs during first gestation in preparation for lactation (Tucker, 1987). Significant growth also occurs in dry cow mammary glands between lactations, contributing to the observed increased milk yields in successive lactations (Capuco et al., 1997; Capuco and Akers, 1999). Previously, the effects of IMI in non-lactating, non-pregnant, mammary growth quiescent heifer glands were examined, and marked changes in glandular structure such as reduced areas of epithelium and increased mammary stroma were described (Trinidad et al., 1990a). These results demonstrated that IMI negatively affects non-lactating mammary gland structure, which is expected to impact mammary function (future growth and development and milk yields). Despite this recognition that IMI impacts non-lactating mammary glands, no studies have investigated how IMI influences histological or morphometric changes in glands that are rapidly growing and developing.

The mammary growth and development occurring during first gestation and subsequent dry periods is largely attributed to the key pregnancy associated hormones, estradiol and progesterone. Substantial literature describes the pivotal roles that these hormones have in driving mammary epithelial cell proliferation (Woodward et al., 1993) and glandular morphogenesis (Sud et al., 1968;

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Howe et al., 1975; Croom et al., 1976) to support subsequent lactation and have been reviewed previously (Akers, 2017). Given the key role of these hormones, their utility, in a model setting, to stimulate rapid mammary growth and development so that molecular mechanisms involved in mammary growth and development may be elucidated shows promise.

It is logical to suspect that an IMI occurring when mammary parenchyma is rapidly growing and developing, (i.e., in the heifer during late gestation or in the multiparous cow during the second half of the dry period) would be problematic. In short, a key question remains: Is rapid mammogenesis compatible with the immune responses initiated to combat a newly developed IMI, and can the processes of mammogenesis and IMI eradication coexist without consequence? If there are conflicts, how are these manifested? While there are differences in the dynamics between the mammary growth experienced in late gestation heifers (parenchyma expansion into the fat pad) and dry cows during the dry period (cessation of milk secretion, regression, and redevelopment, etc.), both situations entail rapid mammogenesis, driven by estradiol and progesterone, to establish the parenchymal tissue necessary for lactation. In this study, non-pregnant, dry cows, that had undergone a more extensive involution than that typically experienced by a dry cow due to the lack of concurrent pregnancy (Capuco et al., 2002), were injected with estradiol and progesterone to stimulate rapid mammogenesis. Subsequently, animals were challenged with *Staphylococcus aureus* to characterize how the presence of IMI influences mammary morphology. The specific objectives of this study were to quantify total and differential somatic cells in mammary secretions from saline and *Staph. aureus* infused mammary glands and define the infiltration of immune cells into mammary tissues. Additionally, a histological evaluation was applied to characterize mammary tissue structure to determine if IMI influenced mammary development.

3.3. Materials and methods

3.3.1. Animal selection and study design

This work was approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol #15-196). A total of 19 animals were selected from the Virginia Tech milking dairy herd for this study, and included non-pregnant, clinically healthy Holstein or Jersey cows that were being culled for reproductive or production reasons, not for reasons concerning udder health. Selected cows were identified from a larger cohort of milking cows for study inclusion by collecting 3 aseptic quarter foremilk samples, with 1

day between each sampling (Dohoo et al., 2011) during the last week of lactation for bacterial examination and SCC quantification. Bacterial examination followed the methods outlined by the National Mastitis Council (Hogan et al., 1999) in which a 10-μL aliquot of fresh milk was streaked onto blood agar plates (Columbia Blood Agar, Hardy Diagnostics, Santa Maria, CA, USA). A local Dairy Herd Information Association Laboratory quantified milk somatic cells using a Fossomatic™ FC (FOSS North America, Eden Prairie, MN, USA). Quarters were classified as infected if 2 of the 3 samples taken were culture positive for the same pathogen (Andersen et al., 2010).

To be included in the study, cows must have had at least 2 uninfected quarters. Furthermore, cows producing foremilk SCC ≤ 200,000/mL for all quarter samplings were preferentially selected over cows yielding higher SCC. Cows were identified and dried off in groups due to limited animal availability. The first group, Group A, contained 6 cows; Groups B and C contained 9 and 4 cows, respectively (Table 3.1). All quarters of all cows were aseptically infused via the partial insertion technique (Boddie and Nickerson, 1986) with a commercial dry cow therapy product (ToMORROW®, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA) at dry-off and then moved to pasture. At 35 days dry, cows were administered a single dose of dinoprost tromethamine (Zoetis, Parsippany, NJ, USA) to synchronize animals to a similar day of estrus and relocated to a sawdust bedded barn. All quarters were aseptically sampled again at 39, 41, and 43 days dry for bacterial examination to confirm that at least 2 quarters were free of IMI.

Cows began a mammary growth induction protocol at 45 days after dry off. The first day of this induction protocol marks the beginning of the core experimental approach used in this trial and will hereafter be referred to as day 1. Cows received consecutive daily injections of estradiol and progesterone on days 1 through 7 as described below to stimulate mammary growth and development. Cows were aseptically sampled again on days 8 and 9 to confirm that mammary glands remained culture negative. Two culture negative quarters from each cow were randomly assigned to receive either an intramammary infusion of sterile phosphate buffered saline (PBS) (n = 19) or *Staph. aureus* (n = 19). Secretion samples were taken immediately before intramammary infusion on day 10, and then again on days 11, 12, 13, 14, 16, 18, and 20 for bacterial examination and quantification and differentiation of somatic cells. During this sampling period, cows were randomly selected for euthanasia at either 5 days post-challenge (day 15; n = 10) or 10 days post-challenge (day 20; n = 9) for collection of mammary tissues.

3.3.2. Estradiol and progesterone injections

Cows were administered daily subcutaneous injections of estradiol (0.1 mg/kg BW; Sigma-Aldrich Co., St. Louis, MO, USA) and progesterone (0.25 mg/kg BW; Sigma-Aldrich Co.) on alternating sides of the neck on days 1 through 7 (Ball et al., 2000). Estradiol and progesterone were dissolved in absolute ethanol, mixed with benzyl benzoate, and sterilized using a 0.45-μm filter. The filtrate was then mixed with autoclaved corn oil, which served as the main injectable carrier. The final injectable solution, containing dissolved estradiol and progesterone, was 10% ethanol, 20% benzyl benzoate, and 70% corn oil by volume.

3.3.3. Mammary secretion sampling and examination

Mammary secretion samples were obtained from cows by removing gross debris from teats and the base of the udder via a single-use paper towel. Teats were dipped in a commercial iodine teat disinfectant (TEAT-KOTE 10/III, GEA United States, Colombia, MD, USA), which remained on teat skin for at least 30 seconds (Enger et al., 2015) before removal with a single-use paper towel. Teat ends were scrubbed with 10 x 10 cm^2 cotton squares soaked in 70% ethanol, and mammary secretions were aseptically expressed into sterile 5-mL round bottom polystyrene tubes. Secretions were immediately placed on ice and transported to the laboratory for culture and somatic cell quantification and differentiation.

Secretion samples were first processed for bacteriological examination using the methods discussed earlier and then used to determine somatic cell concentration (cell/mL) and then differentiate somatic cells. Somatic cells were quantified using the methods outlined by the National Mastitis Council Subcommittee on Screening Tests (Brazis et al., 1968). Fresh secretion samples were first diluted either 1:4 (Group A) or 1:10 (Group B and C) in PBS containing 2.2% bovine serum albumin (BSA). Duplicate smears were prepared by spreading 10 μL of the diluted secretion within a 1-cm^2 circle on milk somatic cell counting slides (Bellco Glass Inc., Vineland, NJ, USA) and then dried at 45°C on a slide warmer. Smears were subsequently stained for 2 min by flooding the slide with Newman's Modified Stain Solution (Sigma-Aldrich Co.). Slides were drained of excess stain, dried, and rinsed in 3 changes of tap water. Stained smears were visualized under oil immersion with a 5-mm square reticle (Microscope World, Carlsbad, CA, USA), which produced a countable strip width of 0.050 mm for the microscope and reticle combination. Stained cells were enumerated by counting the number of cells across the diameter of the circle (11.28 mm) within

the defined strip width. Each smear was counted by 2 independent counters; thus, a total of 4 counts were completed per secretion sample. Enumerated smears were used to calculate the SCC of the undiluted secretion sample, and these SCC were averaged to produce a single SCC estimate for each secretion sample. Final secretion SCC were log_{10} transformed.

The procedures used to differentiate secretion somatic cells were adapted from those described previously (Williams et al., 2017). Briefly, 10 µL of fresh secretion was loaded into the top and bottom chambers of a double cytocentrifuge funnel, and 70-µL of PBS containing 2.2% BSA was added to each chamber. The cytocentrifuge funnel, fitted with a slide, was centrifuged at 110 x g in a Shandon CytoSpin 2 (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min, which produced duplicate smears of the same sample on each slide. Slides were dried at room temperature then stained with a Wright-Giemsa stain (Electron Microscopy Sciences, Hatfield, PA, USA) for 2.5 min. Slides were then drained of excess stain, placed in a stain primed phosphate buffer (6.8 pH; Electron Microscopy Sciences) for 4 min, rinsed with deionized water, and dried again at room temperature. Stained slides were coverslipped and somatic cells were visualized and differentiated by a single operator and classified as being either: 1) neutrophils; 2) macrophages, which would have included any epithelial cells present; or 3) lymphocytes. A total of 100 cells were differentiated for each duplicate smear resulting in 200 cells being differentiated and used to calculate percentages for each cell type.

3.3.4. Intramammary challenge

The *Staph. aureus* Novel strain (Smith et al., 1998) was used as the challenge organism because of its demonstrated ability to induce apoptosis of bovine mammary epithelial cells in vitro (Bayles et al., 1998). Briefly, a single colony of *Staph. aureus* Novel was removed from a Columbia Blood Agar plate that had been incubated for 24 h and placed in a flask containing trypticase soy broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The inoculated flask was incubated at 37°C for 6 h in a shaking incubator rotating at 250 rpm (Kelsey et al., 2006). At the end of the incubation, the bacterial culture was centrifuged at 1,600 x g for 10 min at 4°C. The resulting pellet was resuspended in sterile PBS and washed twice more using these same procedures. The final bacterial pellet was resuspended in sterile PBS to a concentration of 5 x 108 colony forming units (CFU)/mL based on absorbance measured at 600 nm. The adjusted *Staph. aureus* Novel suspension was serially diluted with sterile PBS to a target concentration of 5,000 CFU/mL. For intramammary

infusion, 1 mL of the final dilution was aseptically loaded into tuberculin syringes and transported to the farm on ice. The actual number of *Staph. aureus* Novel CFU infused into mammary glands was confirmed by diluting and plating the final dilution on trypticase soy agar (Becton, Dickinson and Company) and is reported for each group of cows in Table 3.1.

In preparation for infusion, gross debris was removed from teats and the base of the udder via a disposable paper towel, and teats were disinfected using a commercial aerosol teat disinfectant (Fight Bac, Deep Valley Farm Inc., Brooklyn, CT, USA). Teats were dried using a single use paper towel after allowing a teat disinfectant contact time of at least 30 s, and teats ends were then scrubbed with 10 x 10 cm^2 cotton squares soaked in 70% ethanol. Aseptic secretion samples were collected and teats were cleaned again using the commercial aerosol teat disinfectant and cotton squares used before infusion. All quarters were infused via the partial insertion method using sterile teat cannulas (Jorgenson Labs, Loveland, CO, USA) affixed to loaded syringes. Quarters assigned to the saline treatment were always infused before *Staph. aureus* challenge quarters. Gloves were changed between cows and if gloves became soiled.

3.3.5. Tissue collection and processing

Cows were euthanized by captive bolt and exsanguination for tissue collection. Udders were labeled for orientation, removed, cleaned of gross debris using paper towels, and placed on aluminum dissecting trays with the teats facing upward for dissection. Mammary parenchyma tissues were collected from saline and *Staph. aureus* infused quarters. Mammary tissues were collected from parenchyma proximal to the teat, dorsal to the gland cistern for histological evaluation and fixed in 10% formalin for 72 hr. Formalin fixed tissues were transferred and stored in 70% ethanol before being dehydrated in a graded ethanol series and embedded in paraffin using an automated tissue processor (Leica TP 1020; Leica Biosystems Inc, Buffalo Grove, IL, USA).

3.3.6. Tissue histologic analysis

Paraffin embedded mammary tissues were sectioned 5 μm thick using a rotary microtome (Model HM 340 E, Microm International GmbH, Waldoff, Germany) and floated in a water bath at 42°C. Relaxed sections were mounted on Superfrost™ Plus microscope slides (Thermo Fisher Scientific), drained, and dried at 37°C for 24 h on a slide warmer. Slides were deparaffinized in 3 changes of a xylene substitute (Clear-RiteTM 3, Thermo Fisher Scientific) for 5 min each and rehydrated to deionized water using a graded ethanol series. Sections were subsequently stained

with hematoxylin and eosin and coverslipped using the procedures described previously (Tucker et al., 2016).

A single hematoxylin and eosin stained section for each experimental quarter was visualized in its entirety, and 8 representative lobules were identified and imaged at 100x to capture a representative profile of the lobules in each section. Areas of interlobular stroma were avoided, and only lobules were imaged; this was done to focus this analysis on the functional epithelium within the mammary gland. Imaged lobules were classified by a scorer blinded to treatments using a graded scoring scale to characterize the degree of immune cell infiltration in intralobular stroma and luminal area compartments independently. Intralobular stroma infiltration scores ranged from 1 to 4; a score of 1 was a lobule with no evidence of immune cell infiltration (Figure 3.1, score 1) and a score of 4 was a lobule that was more than 2/3 invaded by immune cells (Figure 3.1, score 4). Luminal infiltration scores ranged from 1 to 3; a score of 1 was a lobule that had scant infiltration of immune cells into luminal spaces (Figure 3.2, score 1) and a score of 3 reflected a lobule exhibiting marked luminal infiltration where almost all lumens contained immune cells (Figure 3.2, score 3).

A second examination of these same imaged lobules was conducted to measure the area occupied by different tissue structures, e.g., epithelium, intralobular stroma, and lumen. Lobule and intralobular tissue structures were traced and measured using Image-Pro Plus 7.0 (Media Cybernetics, Rockville, MD, USA; Figure 3.3). This was achieved by first tracing and measuring the area of an imaged lobule and subsequently tracing and measuring the epithelial tissue structures contained within the lobule as they interface with the intralobular stroma. Therefore, these epithelial tracings would include the epithelial structure itself and any luminal areas contained within the structure. Finally, lumens alone, within the original traced lobule and epithelial structures, were traced, measured, and recorded. These tissue areas were used to calculate the epithelial area alone and the intralobular stromal area for each lobule.

3.3.7. Statistical analysis

Total secretion SCC were analyzed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) using $\log_{10}$ transformed SCC as the dependent variable. The final model included the fixed, independent effects of quarter treatment (n = 2), trial day when secretion was collected (n = 8), and their interaction. Cow nested within day of euthanasia and cow nested within day of euthanasia interacting with quarter treatment were included as random effects. Group of cows (n =

3) was not included as a random effect because it was non-significant when tested as a fixed effect ($P > 0.05$). Total $\log_{10}$ SCC were analyzed using a repeated measures approach where trial day served as the repeated time point and cow nested within day of euthanasia interacting with quarter treatment was defined as the measure repeated. Least squares means estimated by the model were compared using a slice procedure to determine if differences existed between treatments within the days secretions were collected.

Differential cell counts were also analyzed using the MIXED procedure of SAS using either, neutrophil, macrophage, or lymphocyte cell type percentages as the dependent variable. Each cell type was analyzed in a separate model. The models used to analyze the respective cell types were identical to the model used to analyze the total secretion SCC. A slice procedure was again used to compare the estimated least squares means for each treatment within day of secretion collection.

Immune cell intralobular stromal invasion and luminal invasion scores were averaged across the 8 representative lobules imaged to obtain mean scores for each experimental quarter sampled. Intralobular stroma and luminal invasion scores served as the dependent variables in two separate models using the MIXED procedure. Both models included the fixed, independent effects of quarter treatment (n = 2) and day euthanized (n = 2); the interactive term of quarter treatment and day euthanized was not included based on non-significance ($P \geq 0.30$). Cow nested within day of euthanasia was specified as a random effect in both models, but group of cows (n = 3) was removed as a random effect because it was non-significant in all models when tested as a fixed effect ($P \geq 0.15$). Resultant least squares means were contrasted using Fisher's least significant differences test.

Measured tissue areas were used to calculate the percentage of lobule area occupied by: 1) intralobular stroma; 2) epithelial structures; and 3) luminal space for each lobule. These measures were subsequently averaged across the 8 representative lobules imaged to obtain mean percentages for each experimental quarter sampled. Intralobular stroma, epithelial structure, and luminal space percentages served as dependent variables in 3 separate models, which used the MIXED procedure. These models were identical to those previously described and used to analyze tissue immune cell invasion scores. Least squares means estimated by the models were contrasted using Fisher's least significant differences test.

3.4. Results

3.4.1. Success of challenge

Intramammary *Staph. aureus* Novel challenge established IMI in 18 of the 19 infused quarters. All *Staph. aureus* infections persisted until tissues were collected at either 5 or 10 days post-challenge, and all saline infused quarters remained culture negative throughout. Challenged quarters did not display clinical signs of inflammation, such as quarters being red, swollen, or hot to the touch, but small flakes were occasionally observed in challenged quarter secretions. Secretion and tissue samples collected from the cow that did not develop an IMI in the challenged quarter were not utilized in any of the preceding described analyses. As a result, secretions and tissues were examined for 9 cows that were euthanized 5 days post-challenge and 9 cows euthanized 10 days post-challenge.

3.4.2. Secretion somatic cells

The mean secretion *Staph. aureus* quarter SCC (7.45 ± 0.06 $\log_{10}$ cells/mL) was greater than the mean saline quarter SCC (6.77 ± 0.06 $\log_{10}$ cells/mL; $P < 0.001$). Additionally, secretion SCC were significantly influenced by treatment interacting with trial day ($P < 0.001$; Figure 3.4, Panel A). Overall, secretion SCC appeared unchanged in saline quarters throughout the trial's duration (Figure 3.4; Panel A) and these SCC were significantly lower for all days sampled post-challenge relative to challenged quarters ($P < 0.05$).

Neutrophil, macrophage, and lymphocyte percentages measured in saline and challenged quarter mammary secretions are stratified by day of trial and are illustrated in Panels B-D in Figure 3.4; representative images of each cell type are shown in Figure 3.5 (Panels A and B). Overall, the mean percentage of neutrophils in challenged quarters ($47.2\% \pm 2.3$) was greater than the mean neutrophil percentage in saline quarters ($7.1\% \pm 2.3$; $P < 0.001$); conversely, the mean percentages of macrophages and lymphocytes in challenge quarters ($17.7\% \pm 3.0\%$ and $34.4\% \pm 2.1\%$, respectfully) were lower than those measured in saline quarters ($51.0\% \pm 3.0\%$ and $40.1\% \pm 2.1\%$, respectfully; $P \leq 0.03$).

Aside from these main effects, a significant interaction existed between quarter treatment and trial day in its effect on all the measured cell type percentages ($P < 0.001$). In general, saline quarter cell type percentages remained stable throughout the trial, but cell type percentages in *Staph. aureus* quarters changed in response to challenge. For instance, neutrophil percentages

were greater for every day sampled post-challenge in challenged quarters compared to saline quarters ($P < 0.001$; Figure 3.4, Panel B) and this appeared to impact macrophage percentages, which were lower in challenge quarters for every day sampled post-challenge ($P < 0.001$; Figure 3.4, Panel C). In addition, some neutrophils collected from challenge quarters in the present study were observed to contain intracellular *Staph. aureus* (Figure 3.5, Panel B). Lymphocyte percentages were lower in challenged quarters than saline quarters for the first 3 days following challenge ($P < 0.05$), but not for the remainder of the days sampled ($P > 0.05$; Figure 3.4, Panel D).

Eosinophils were also observed in secretions (Figure 3.5, Panel A) but made up less than 1% of the differential cell count, preventing comparisons from being made between saline and challenged quarters. Binucleated giant cells and lumen resident cells undergoing mitosis were also sporadically observed (Figure 3.5, Panel C and D).

3.4.3. Tissue measures

Immune cell infiltration scores were not affected by day of euthanasia ($P \geq 0.25$) but were greater for challenged quarter lobules than saline quarter lobules for both the luminal (1.68 vs 1.13 ± 0.09; $P < 0.001$) and intralobular stroma compartments (1.85 vs. 1.50 ± 0.09; $P = 0.005$) (Figure 3.6). Saline quarter lobules were essentially devoid of neutrophils in both the luminal and intralobular stromal compartments (Figure 3.7, Panel A), but neutrophils were frequently observed in both compartments of *Staph. aureus* challenged quarter lobules (Figure 3.7, Panel B). Lymphocytes could be observed in both saline and challenged quarters but were more abundant in the latter. It is noteworthy to state that lymphocytes appeared to preferentially accrue in intralobular stromal compartments rather than luminal spaces (Figure 3.7, Panel C). Plasma cells were abundant in both saline and challenged quarter tissues, but did not grossly appear to be more abundant in one versus the other (Figure 3.7, Panel D).

Lobules in *Staph. aureus* challenged quarters exhibited a greater percentage of luminal space (7.7% vs 5.4% ± 0.6%; $P = 0.004$), a reduced percentage of epithelial area (33.3% vs 38.1% ± 1.1%; $P < 0.0001$), and tended to have a greater percentage of intralobular stromal area (59.0% vs 56.5% ± 1.3%; $P = 0.1$) than saline infused glands (Figure 3.8).

3.5. Discussion

3.5.1. Secretion somatic cells

The first objective of this study was to characterize the somatic cell and differential cell count response resulting from infusion of saline and *Staph. aureus* Novel into non-lactating mammary glands. The absence of an increase in secretion SCC in saline infused quarters was expected and indicates that no significant immune response resulted from saline infusion and complements the observation that saline quarters remained culture negative throughout the trial. Conversely, the significant increase in secretion SCC observed in response to *Staph. aureus* challenge was expected and establishes that an immune response resulted in these quarters. No reports could be identified describing the SCC response of non-lactating mammary glands to *Staph. aureus* challenge but it has been reported that secretions collected from uninfected quarters of pregnant dry cows, contain a lower SCC than those collected from infected quarters (Jensen and Eberhart, 1981). Furthermore, the reported SCC of uninfected and infected quarters in the previous report (Jensen and Eberhart, 1981) are comparable to the SCC observed here, indicating that the SCC response was similar to that of the pregnant dry cow. Aside from the SCC response in non-lactating glands, previous reports in lactating cows (Nickerson, 1980; Schukken et al., 1999; Middleton et al., 2004) have described increases in SCC resulting from *Staph. aureus* challenge.

The observed increase in neutrophil percentages in *Staph. aureus* infused quarters were expected given neutrophils are the main innate immune effector cell in the bovine mammary gland that respond to IMI (Paape et al., 2002) and similar changes have been described in other challenge trials in heifers (Jackson et al., 2012) and lactating cows (Nickerson, 1980). Neutrophils have also been described as being the predominate cell type in infected dry cow gland secretions (Jensen and Eberhart, 1981). Lymphocytes were the second most predominate cell type observed in challenged quarters but, this observation is not consistent with two previous reports (Jensen and Eberhart, 1981; Sordillo et al., 1987) that reported that macrophages were the second most predominant cell type infected dry cow secretions. Reasons for this disparity are unclear but are posited to be attributed to the fact that the secretions collected in those two previous studies were from quarters naturally and chronically infected with an assortment of different mastitis pathogens. The secretions collected herein were collected from quarters responding to a *Staph. aureus* challenge and are thus more associated with a rapid immune response rather than an immune response

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linked to chronic infections. Also, the immune response generated herein was specific to *Staph. aureus* and it has been previously demonstrated that different mastitis pathogens elicit different cytokine profiles and immune responses (Bannerman et al., 2004) which would, in part, explain the discrepancy between these results.

Binucleated giant cells, like those observed here, have been reported and discussed previously (Nickerson and Sordillo, 1985; 1987; Williams et al., 2017), but how these cells originate in the gland is not entirely clear. It is possible that a subset of these cells originated from what appear to be lumen resident macrophages undergoing incomplete cell division (Figure 3.5, Panel D) given that these mitotic cells were most commonly observed within secretions containing binucleated cells. Additionally, binucleated epithelial cells have also been described (Rios et al., 2016) and would contribute to the presence of these cells in milk or mammary secretions should they be sloughed from the basement membrane into the lumen.

3.5.2. Tissue measures

The second key objective of this study was to characterize mammary tissue structure in quarters infused with saline and *Staph. aureus* to define how *Staph. aureus* challenge affected mammary gland structure and development. The observed infiltration of immune cells into *Staph. aureus* infected tissues was expected and complements the influx of immune cells into challenged quarter mammary secretions that would result from leukocyte diapedesis from blood vessels to mammary gland lumens in response to the presence of *Staph. aureus*. The abundance of plasma cells observed in mammary tissues from both treatments is believed to be consequence of the estradiol and progesterone injections given their significance in colostrogenesis (Weisz-Carrington et al., 1978; Barrington et al., 2001). Not surprisingly, a similar hormonal induction model to that used here has been used to investigate bovine colostrogenesis mechanisms (Stark et al., 2015). Examination of colostrum formation and immunoglobulin transport was not an objective in the present study but the abundance of plasma cells in the collected tissues may warrant consideration for future studies investigating colostrogenesis mechanisms, particularly those concerned with immunoglobulin production and transport.

No reports examining the histopathological response of a mastitis challenge in non-pregnant, dry cows could be identified with which to compare the tissue area percentages reported here. However, a previous report described the histopathological response of mammary tissue in

non-pregnant heifers after *Staph. aureus* challenge (Trinidad et al., 1990a) and reported a similar reduction in the epithelial areas relative to uninfected quarters. This previous study also reported that *Staph. aureus* infected tissues contained greater areas of stroma tissue than uninfected quarters and complements the tendency of *Staph. aureus* quarter lobules to contain greater areas of intralobular stroma than saline lobules as similarly reported here. Differing from the results of this previous study was the observation that challenged quarters exhibited larger luminal areas than saline infused quarters. The larger luminal areas observed herein are suspected to be consequence of the initial immune cell influx into the gland's lumen, bringing fluid across the epithelium, given that IMI reduces epithelial integrity and results in increased concentrations of BSA (Chockalingam et al., 2005) and ions (Linzell and Peaker, 1972) in milk from affected quarters. The reason for the lack of agreement between the previous study and the results of the present may also be attributed to differences in infection duration. The cows used here were euthanized 5 and 10 days post challenge, whereas the previous study euthanized heifers 2 to 3 weeks post challenge (Trinidad et al., 1990a). This longer infection duration would have allowed the sustained immune response to affect glandular structure over a longer period of time. As a result, continued deposition and accumulation of connective tissues, leading to fibrosis, would result and begun to displace luminal space as fluid from infected quarters was reabsorbed, given the initial immune response had begun to subside.

The dry cows used in this study were treated with estradiol and progesterone to stimulate mammary growth and development so that IMI impact in growing and developing mammary glands could be investigated. In this context, the reductions in epithelial areas and tendency for challenged glands to contain greater areas of stromal tissue indicate that challenged glands failed to develop comparable amounts of epithelium and experienced varying degrees of connective tissue deposition in the gland as a result of IMI. It is currently unknown what chief mechanisms are responsible for these changes in glandular structure, but the deposition and accumulation of connective tissues in affected tissues, displacing mammary epithelium, and the immune response produced to address the presence of bacteria may interfere with mammary epithelial cell proliferation and alter gland development, perhaps in the long term. Such changes in glandular development are expected to contribute, in part, to the reduced milk yields reported for heifers that freshen with IMI (Owens et al., 1991; Oliver et al., 2003) as well as the reduced milk yields

described for cow quarters that freshen with IMI compared to paired, uninfected, lateral quarters within the same cow (Smith et al., 1968).

Interestingly, neither day of euthanasia for tissue collection nor the interaction between day of euthanasia and treatment significantly influenced any of the respective lobule area percentages measured ($P \geq 0.18$). This was unexpected, but is not entirely surprising given this study's experimental design. This study was designed to first allow for treatment comparisons to be made within animal to control for inter-animal variation. When an examination of day of euthanasia was applied to this design, resulting in the nesting of animals within day euthanized, control for between animal variation was lost, which significantly influenced the studies ability to detect quarter treatment differences between animals euthanized at 5 or 10 days post challenge. Furthermore, perhaps examining tissues 5 days post-challenge was too late to capture the temporal changes occurring in gland morphology resulting from intramammary *Staph. aureus* challenge; collection of tissues closer to the initial challenge may have allowed for changes over time in gland structure to be better appreciated.

3.6. Conclusions

The results of this study indicate that *Staph. aureus* challenge of rapidly growing, non-lactating mammary glands increases immune cell invasion in mammary secretions and both the intralobular stroma and luminal compartments of the mammary gland. This invasion was associated with changes in mammary structure as *Staph. aureus* challenged quarters exhibited reduced areas of epithelium and tended to have greater areas of intralobular stroma relative to saline infused quarters. When these histological changes are taken together, it suggests that IMI in rapidly growing non-lactating mammary glands limit mammary growth and development, which is expected to negatively impact future milk yield, milk quality, and productivity of the animal in the herd.

3.7. Acknowledgments

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Table 3.1. Experimental animal group demographics and corresponding *Staph. aureus* Novel inoculums infused into challenge quarters.

Group	Breed	Mean Last Week Milk SCC (cells/mL)	Lactations Completed	Mean Days in Milk	Mean Last Week Milk Yield (kg/day)	*Staph. aureus* inoculum (CFU)
Group A	Holstein (n = 6)	141,000	1 (n = 5)	568	25.7	4,300
			3 (n = 1)			
Group B	Holstein (n = 6); Jersey (n = 3)	126,000	1 (n = 7)	391	28.4	6,400
			2 (n = 1)			
			6 (n = 1)			
Group C	Holstein (n = 4)	251,000	1 (n = 2)	611	22.0	7,500
			2 (n = 1)			
			4 (n = 1)			
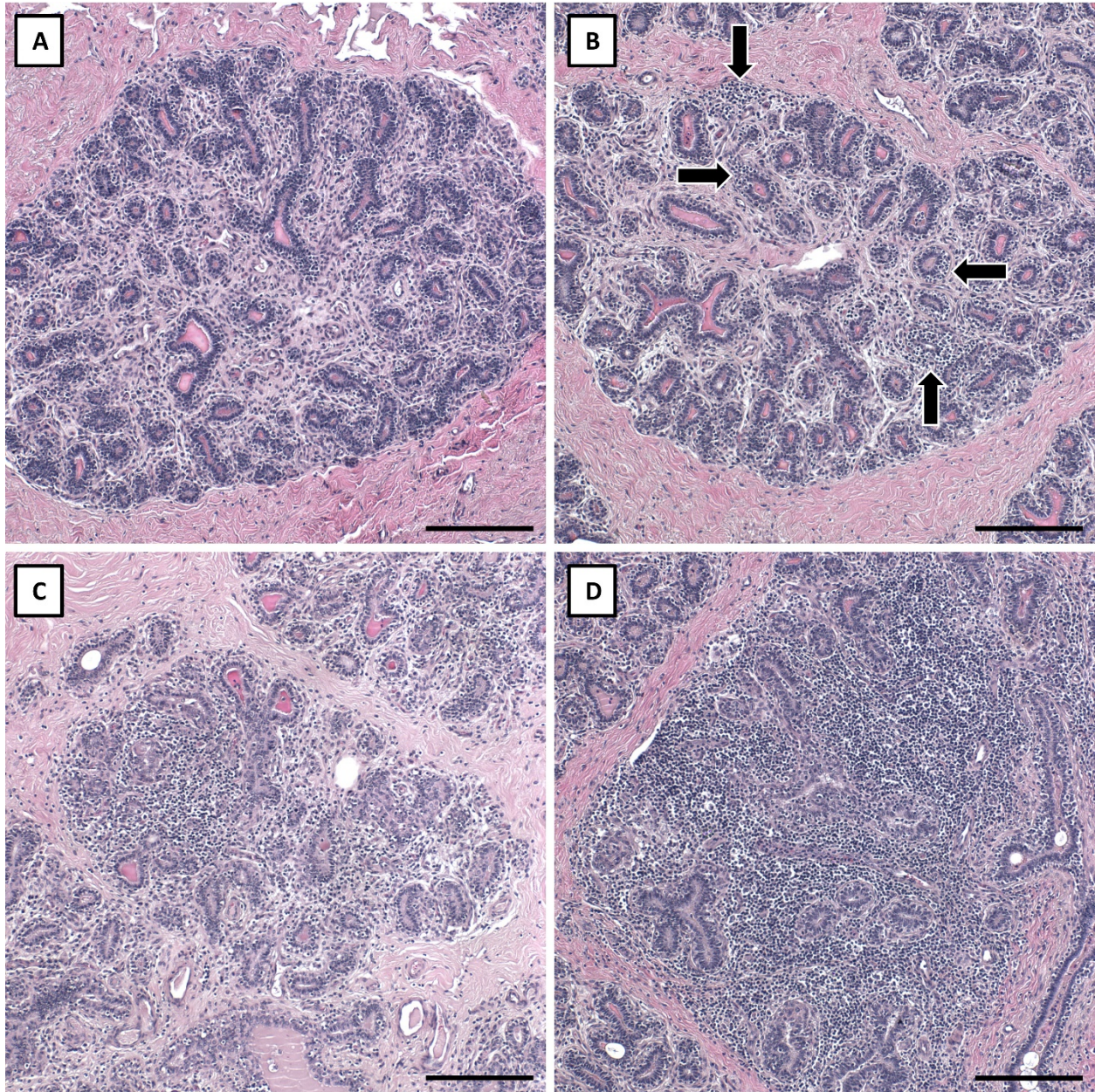
Figure 3.1. Intralobular stroma immune cell invasion scoring. The presented quarter lobules were used to characterize the degree of intralobular stromal immune cell invasion observed in saline and *Staph. aureus* quarter lobules; scores 1 - 4. Panel A depicts a score of 1 with no infiltration; Panel B depicts a score of 2 where small isolated pockets of infiltration (arrows) are present; Panel C exemplifies a score of 3 where cellular infiltration affecting approximately 1/3 of the lobule; and Panel D depicts a score of 4, marked infiltration affects more than 2/3 of the lobule. Scale bars = 200 μm.
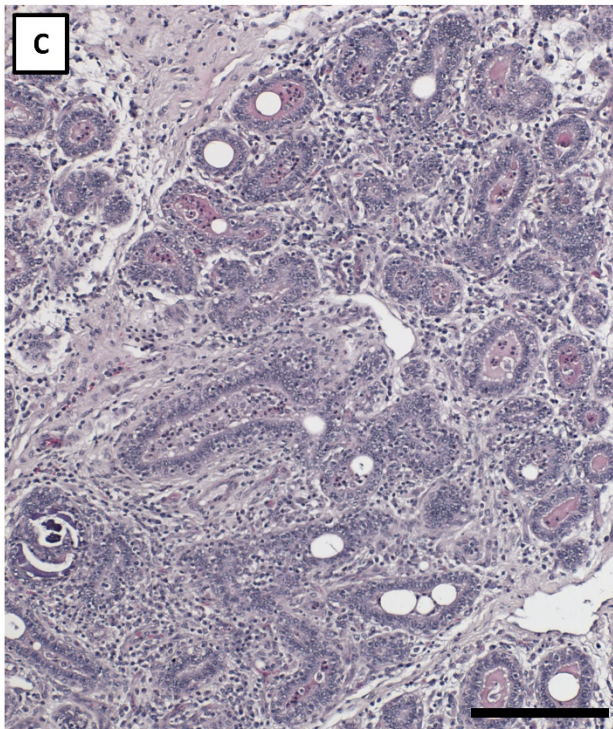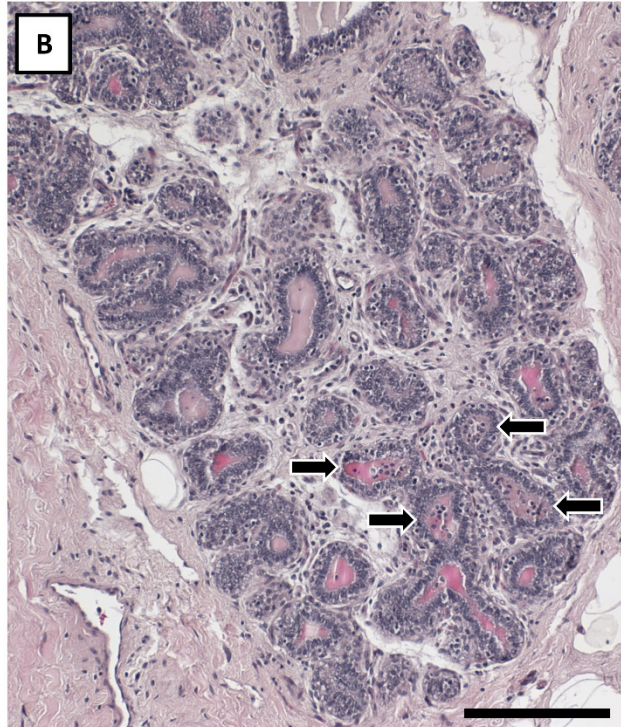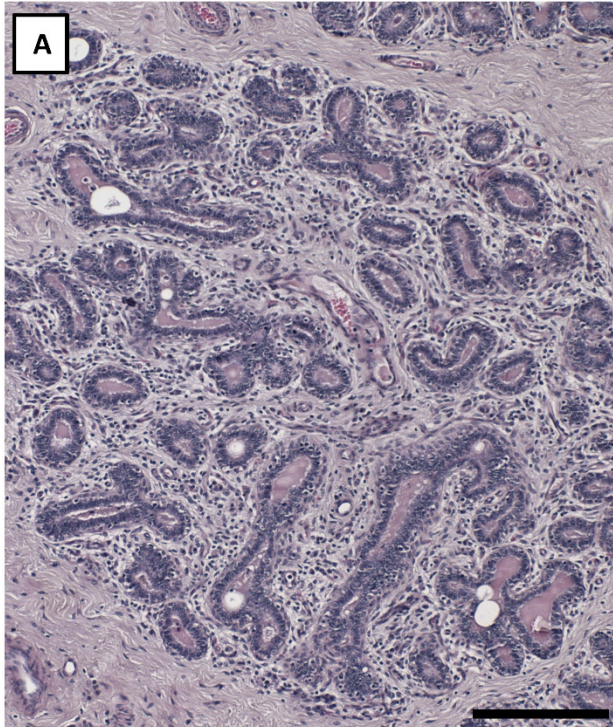
Figure 3.2. Luminal immune cell invasion scoring. The presented quarter lobules were used to characterize the degree of luminal immune cell invasion observed in saline and *Staph. aureus* quarter lobules; scores 1- 3 are presented. Panel A depicts a score of 1 which signifies no luminal infiltration; Panel B depicts a score of 2 with infiltration in fewer than half the lobule lumens (arrows); and Panel C denotes a score of 3 which is marked infiltration in most lumens. Scale bars = 200 μm.
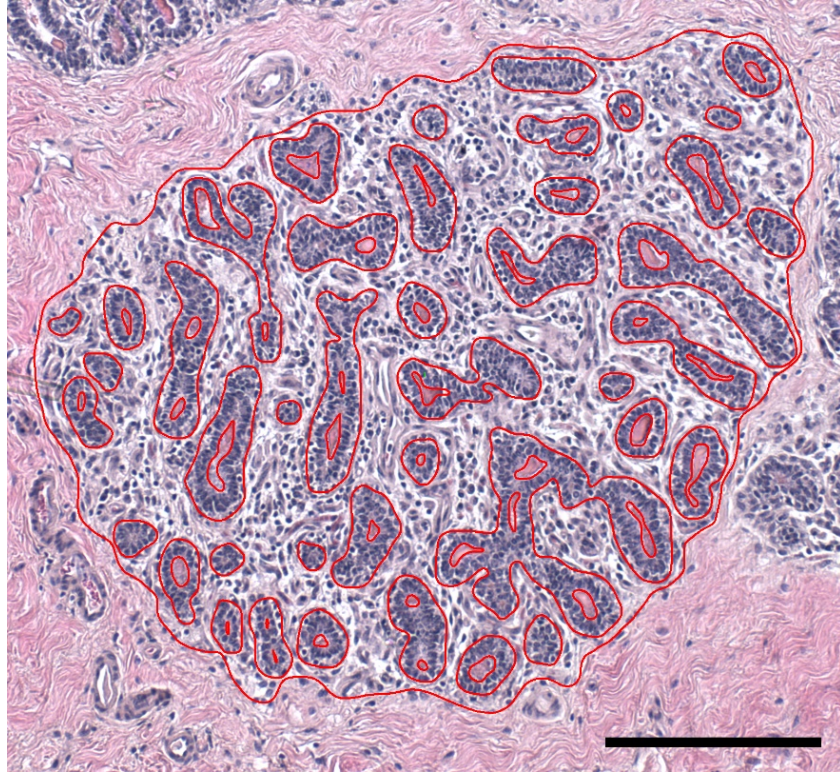
Figure 3.3. Measurement of tissue area percentages in imaged lobules. Example image of the tracings applied to imaged lobules to measure lobule, intralobular stroma, epithelial, and luminal areas. Scale bar = 200 μm.
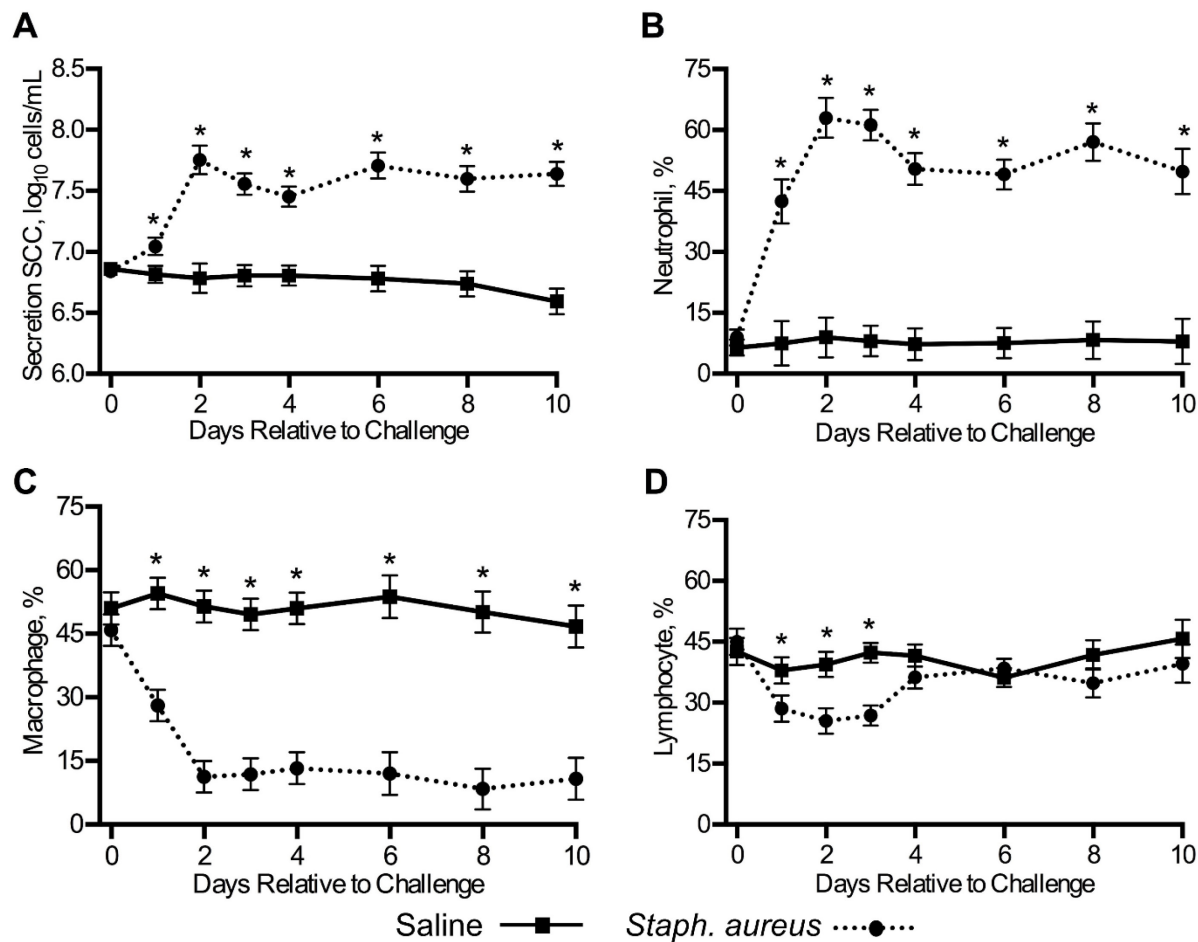
Figure 3.4. Total SCC and differential cell type percentages in collected mammary secretions. Total secretion SCC (Panel A) and corresponding differential cell type percentages (Panels B-D) collected from saline (n = 18) and *Staph. aureus* (n = 18) infused quarters. Error bars represent the standard error of the respective means. *$P \leq 0.05$.
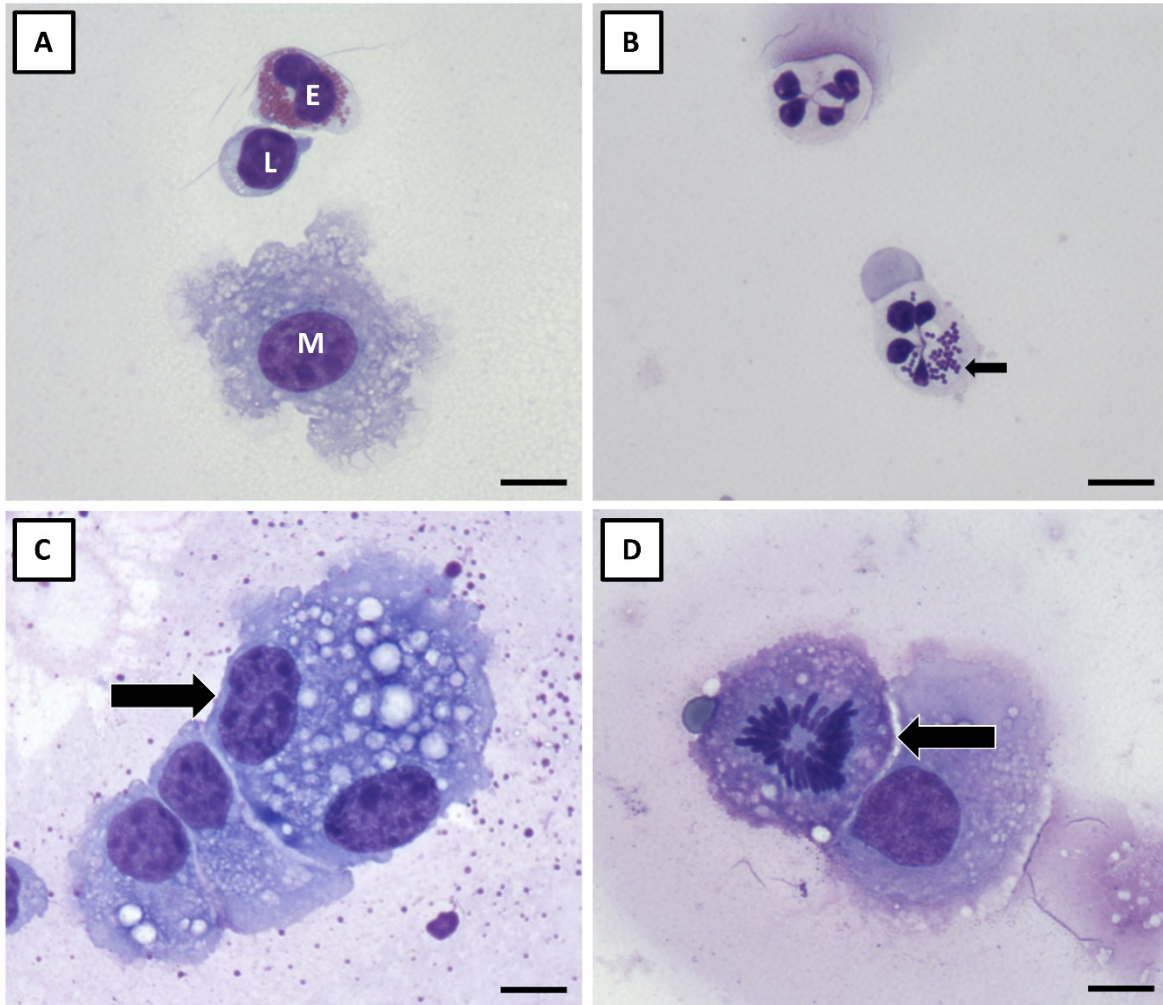
Figure 3.5. Somatic cells observed in collected mammary secretions. An eosinophil (E), lymphocyte (L), and macrophage (M) are depicted in Panel A. Panel B depicts two neutrophils collected from a challenged quarter with the bottom neutrophil containing intracellular *Staph. aureus* (arrow). A binucleated macrophage is shown in Panel C (arrow). These cells are suspected to originate from lumen resident macrophages that undergo incomplete cell division like the mitotic cell (arrow) shown in Panel D. Scale bars = 10 µm.
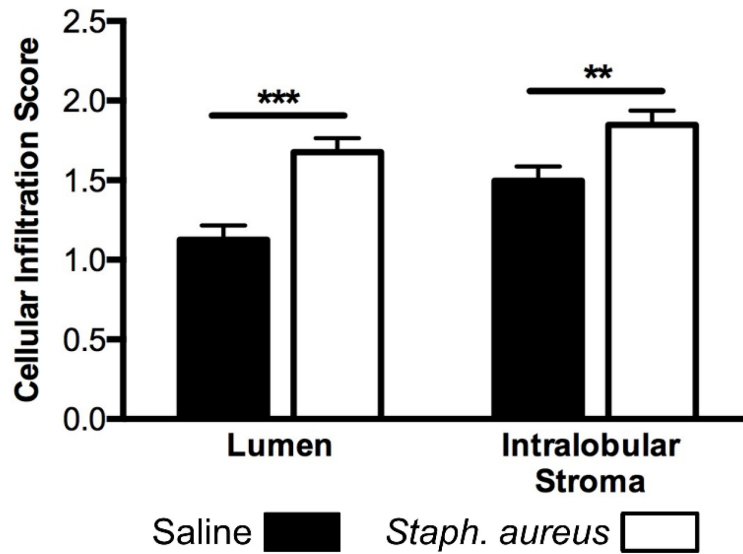
Figure 3.6. Mean immune cell infiltration scores for lumen and intralobular stroma areas. Mammary tissues were collected from 18 saline and 18 *Staph. aureus* infused quarters and 8 representative lobules were scored for each experimental quarter. Error bars represent the standard error of the respective mean immune cell infiltration scores. Asterisks denote differences between saline and *Staph. aureus* quarter treatments within intralobular stroma and luminal areas. **$P \leq 0.01$, ***$P \leq 0.001$.
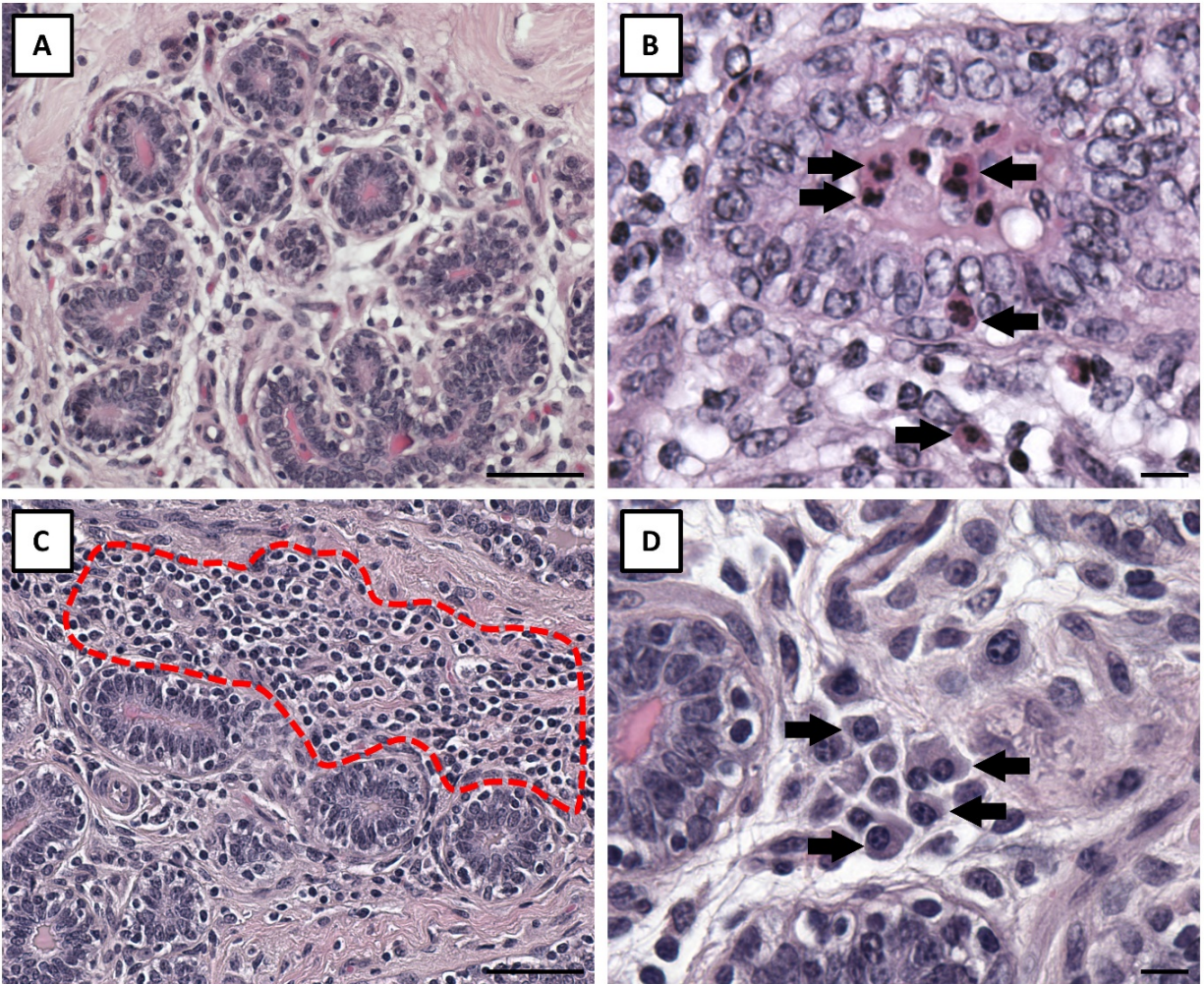
Figure 3.7. Cellularity of tissues from saline and *Staph. aureus* quarter lobules. Panel A depicts tissues from a saline infused quarter exhibiting diffuse intralobular stroma and non-secretory epithelium. Neutrophilic infiltration (arrows) of luminal and intralobular stroma compartments is depicted in Panel B for tissues from a *Staph. aureus* infused quarter lobule. Panel C exemplifies the preferential infiltration of lymphocytes into intralobular stroma areas (dashed outline) that could be observed in saline quarters but were more frequent in *Staph. aureus* quarters. Plasma cells (arrows) could be observed in both saline and *Staph. aureus* quarter lobules (Panel D). Scale bars in A and C = 50 µm, B and D = 10 µm.
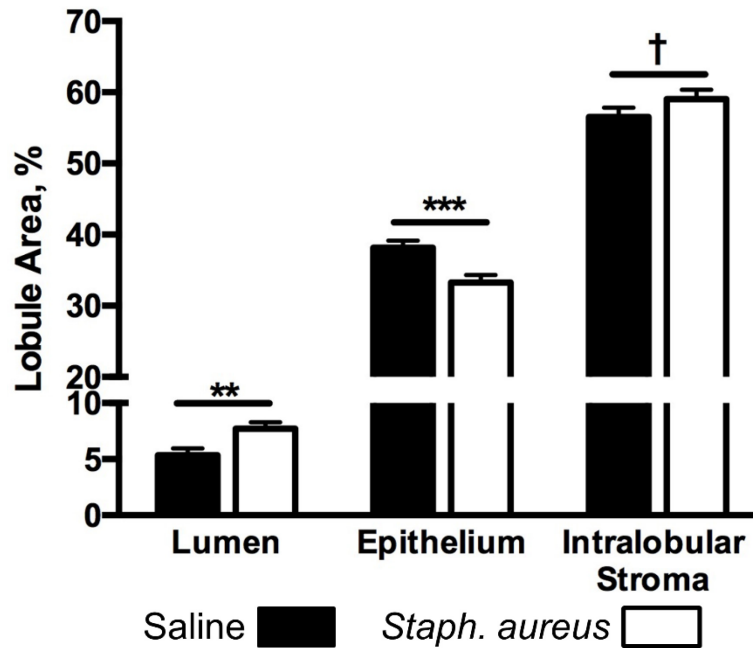
Figure 3.8. Lobule area percentages occupied by luminal space, epithelium, and intralobular stroma in experimental quarters. Mammary tissues were collected from 18 saline and 18 *Staph. aureus* infused quarters and 8 representative lobules from each quarter were used to quantify lobule areas occupied by intralobular stroma, epithelium, and luminal space. Error bars represent the standard error of the respective mean percentages. Dagger symbol and asterisks denote differences between saline and *Staph. aureus* quarter treatments within tissue structures; $^{†}P = 0.1$, $**P \leq 0.01$, $***P \leq 0.001$.

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CHAPTER 4: Apoptosis and proliferation in *Staphylococcus aureus* challenged, non-lactating mammary glands, stimulated to rapidly grow and develop with estradiol and progesterone

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4.1. Abstract

Bovine mastitis is a common and costly disease in the dairy industry and is known to
negatively affect the amount of epithelium in non-lactating mammary glands. Despite this
recognition, an understanding of the mechanisms contributing to reductions in epithelium is
lacking. The objective of this study was to evaluate cellular apoptosis and proliferation in uninfected
and *Staphylococcus aureus* infected mammary glands that were stimulated to rapidly grow and
develop. Estradiol and progesterone injections were administered to 18 non-lactating dairy cows to
induce mammary growth, and 2 quarters from each animal were infused with saline or *Staph.
aureus*. Mammary tissues were collected at 5 (n = 9) and 10 (n = 9) days post-infusion and examined
using quantitative brightfield and florescent immunohistochemistry. *Staph. aureus* mammary
glands tended to have a greater number of mammary epithelial cells undergoing apoptosis than
saline quarters. In the stromal compartment, challenged quarters contained a lower proportion of
cells undergoing apoptosis than saline quarters overall; but, cell types undergoing apoptosis were
differentially affected. *Staph. aureus* quarters contained a lesser percentage of apoptotic fibroblasts
while also containing more non-apoptotic immune cells than saline quarters in the intralobular
stroma compartment. A similar number of proliferating epithelial cells were present in *Staph.
aureus* and saline mammary tissues but more proliferating cells were present in the intralobular
stroma compartment of *Staph. aureus* infused quarters than saline. When these cellular responses
are considered together, it indicates that changes in cellular apoptosis and proliferation contribute
to changes in gland structure by potentiating the expansion of the intralobular stromal
compartment, via cellular accumulation, and limiting the amount of epithelium due to increases in

cellular apoptosis in affected glands. Reductions in mammary epithelium are expected to reduce future milk yields and productive herd life.

4.2. Introduction

Bovine mastitis is almost exclusively the result of a bacterial intramammary infection (**IMI**) and continues to be a common and challenging disease for the US and global dairy industries. It is well recognized that mastitis in lactating mammary glands negatively impacts the synthesis and secretion of milk components as well as mammary structure. For instance, reductions in milk yield and quality occur as milk somatic cell count (a measure of mammary inflammation) increases (Jones et al., 1984; Hogan et al., 2016) and many investigators have previously described (Spencer, 1949; Nickerson, 1980; Akers and Thompson, 1987) and reviewed (Akers and Nickerson, 2011) the marked infiltration of immune cells, fibrosis of affected tissues, and atrophy of secretory tissues relative to non-mastitic glands that result in response to mastitis.

Milk yield is a function of the number of secretory mammary epithelial cells in the mammary gland and their secretory activity; a reduction of either would negatively impact milk yield. In relation to mastitis, it is expected that the pathogens and inflammation implicated in this disease create conditions that would reduce the cell numbers in the gland via apoptosis, and potentially necrosis during acute infections. Previously, Bayles et al. (1998) observed apoptosis of MAC-T cells (a bovine mammary epithelial cell line) when cells were challenged with *Staphylococcus aureus*. Hu et al. (2014) also challenged bovine mammary epithelial cells in vitro with *Staph. aureus* and described activation of the FAS-FADD apoptosis pathway and increased activities of the integral apoptosis enzymes, caspase-3 and 8. Expanding this work into the lactating bovine mammary gland, Long et al. (2001) established that intramammary infusion of *Escherichia coli* also induces apoptosis of mammary epithelial cells. Undoubtedly, induced death of mammary epithelial cells, which would eventually give rise to secretory epithelium and functional alveoli, when the mammary gland is growing would be undesirable and would likely negatively impact future milk yields.

Aside from IMI in lactating animals, it is also recognized that IMI are common in non-lactating heifers (Fox, 2009) and dry-cows (Eberhart, 1986). The occurrence of IMI in these animals is concerning given the considerable amount of mammary growth that occurs during first gestation (Tucker, 1987) and the last half of the dry period (Capuco et al., 1997). The impacts of IMI in the mammary glands of non-pregnant, nulliparous heifers have been previously investigated, and reductions in mammary epithelium and increased stromal tissue areas were described (Trinidad et al., 1990). More recently, Andreotti et al. (2017) investigated the impacts of *Staph. aureus* IMI in

cows during the first 3 weeks of the dry period and observed an increased percentage of epithelial cells undergoing apoptosis in *Staph. aureus* infected quarters relative to uninfected quarters. When these reports are taken together, it is evident that IMI in non-lactating mammary glands negatively impacts mammary gland structure by reducing mammary epithelium and increasing the percentage of mammary epithelial cells undergoing apoptosis. This is expected to reduce future milk yields. Although the results of these two reports (Trinidad et al., 1990; Andreotti et al., 2014) are telling, neither investigates how the mammary gland is impacted by IMI during periods of rapid mammary growth and development such as in the pregnant dairy heifer or during the last half of the dry-period in the cow.

Previously, Enger et al. (2018) examined *Staph. aureus* IMI's impact on mammary tissue structure in glands that were stimulated to rapidly grow and develop, and observed a significant reduction in intralobular areas occupied by epithelium and increased areas of intralobular stroma. It is unknown what chief mechanisms contribute to these changes in tissue structure, but it is posited that a greater number of mammary epithelial cells would be undergoing apoptosis in challenged glands based on prior studies. The objective of this study was to evaluate cellular apoptosis and proliferation, using quantitative histology, in uninfected and *Staph. aureus* infected mammary glands that were stimulated to rapidly grow and develop using estradiol and progesterone injections.

4.3. Materials and methods

4.3.1. Study design

The mammary tissues used here were collected as previously described in detail by Enger et al. (2018). In brief, 19 non-pregnant, non-lactating dairy cows that had been dry for 45 days, were administered 7 consecutive daily injections of estradiol (0.1 mg/kg BW) and progesterone (0.25 mg/kg BW) to stimulate mammary growth and development. Three days after the last injection, 2 culture-negative quarters of each cow were randomly infused with either sterile, physiological, phosphate-buffered saline (n = 19) or *Staph. aureus* (n = 19). All saline infused quarters remained culture negative throughout the trial, and all but one cow developed a *Staph. aureus* intramammary infection in the challenged quarter. At 5 days post-challenge, half of the cows were randomly selected for euthanasia and tissue collection; tissues were collected from the remaining animals at 10 days post-challenge. Mammary parenchyma that was most proximal to the teat, but dorsal to

the gland cistern was collected from experimental quarters. Tissues were fixed and processed as previously described (Enger et al., 2018). The mammary tissues collected from the animal that did not establish a *Staph. aureus* IMI were not utilized.

4.3.2. Immunohistochemistry

Two mammary tissue slide sets were prepared; one was used to examine expression of cleaved caspase-3 (an apoptosis marker) and the other used to examine expression of Ki-67 (a proliferation marker) in the collected mammary tissues. Tissues were sectioned 5 µm thick using a rotary microtome (Model HM 340 E, Microm International GmbH, Waldoff, Germany) and relaxed in a 42°C water bath. For both slide sets, 2 to 3 serial sections, from each experimental quarter were mounted to Superfrost™ Plus microscope slides (Thermo Fisher Scientific, Waltham, MA), drained, and dried at 37°C for 24 h. All sections were deparaffinized in 3 changes of a xylene substitute (Clear-RiteTM 3, Thermo Fisher Scientific) and rehydrated to deionized water using a graded ethanol series before being subjected to different immunohistochemistry protocols.

Cleaved caspase-3 staining: The cleaved caspase-3 slide set was prepared using a bright-field immunohistochemistry kit (Histostain-Plus IHC Kit, Thermo Fischer Scientific). Antigens were retrieved by immersing slides in 10-mM citrate buffer, pH 6.0, for 30 min at 95°C. Slides were removed from the heat source after antigen retrieval and allowed to cool to room temperature while remaining submerged in citrate buffer. Sections were subsequently washed in 0.9% NaCl PBS, pH 7.4, quenched using 3% H_2O_2 for 10 min at room temperature, and washed again with PBS. A hydrophobic barrier pen (Liquid Blocker, Daido Sangyo Co., Ltd, Saitama, Japan) was used to separate tissue sections on the same slide to isolate section specific reagents and antibody mixtures. All sections were blocked using the provided kit reagent for 30 min.

The blocking agent was aspirated from all sections and 100 µL of the blocking reagent was reapplied to a single section on each slide; this section served as the negative control. Remaining sections received 100 µL of the primary antibody mixture that contained the cleaved caspase-3 antibody (Table 4.1) suspended in the kit provided blocking solution and incubated in a dark humidified chamber at 4°C for 16 h. After primary antibody incubation, solutions were aspirated, and sections were washed thrice with PBS for 2 min each washing. All tissue sections were then incubated with 100 µL of the kit provided streptavidin conjugated horseradish peroxidase for 10 min at room temperature before being washed thrice more, 2 min each washing, with PBS. The

horseradish peroxidase streptavidin conjugate was reacted with freshly prepared DAB chromogen for 3 min and immediately washed 3 times with PBS for 1 min each washing. Sections were lightly counterstained by quickly dipping slides in hematoxylin 3 times and rinsing with tap water. Sections were dehydrated, dried, and coverslipped (Permount™, Thermo Fisher Scientific).

Ki-67 staining: Antigen retrieval and isolation of tissue sections for the Ki-67 slide set followed the exact procedures described for the cleave caspase-3 slide set. Differing from the previous slide set, the Ki-67 slide set sections were blocked using a universal blocking agent (CAS Block, Thermo Fisher Scientific) for 30 min.

CAS Block was aspirated from the sections and 100 µL of CAS Block was reapplied to a single negative control section on each slide. The remaining sections received 100 µL of the primary antibody mixture containing Ki-67 antibody (Table 4.1) suspended in CAS Block. Sections were incubated in a dark humidified chamber at 4°C for 16 h. After primary antibody incubation, solutions were aspirated, and sections were washed thrice with PBS for 5 min each washing. All tissue sections were then incubated with 100 µL of a secondary antibody mixture, containing the secondary antibody (Table 4.1), suspended in CAS Block, at room temperature for 60 min. Secondary antibody mixtures were aspirated, and sections were washed again with PBS. Residual hydrophobic barrier pen outlines were removed and sections were coverslipped using ProLong™ Gold Antifade Mountant containing DAPI (Thermo Fisher Scientific). Coverslipping mountant was cured overnight before sections were imaged.

4.3.3. Imaging and image analysis

Cleaved caspase-3 sections: For the cleaved caspase-3 stained sections, 6 images of mammary parenchyma were randomly identified and imaged. Images were acquired with a bright field color camera (Retiga R6, QImaging Corporation, Surrey, BC, Canada) affixed to an Olympus BX43 light microscope (Olympus Corporation of the Americas, Center Valley, PA) with a 40x objective. With the microscope configuration used, the total area imaged in 6 microscopic fields was 0.458 mm^2.

Acquired images were first analyzed by counting the number of positively stained cleaved caspase-3 nuclei within the mammary epithelial and luminal compartments. The respective compartmental counts were summed across the 6 randomly acquired images from each experimental quarter to produce a total number of positive cells across a constant area examined.

This approach is similar to that previously described by Long et al. (2001). Next, for each experimental quarter, a subset of 3 randomly selected images were examined once more so that cells within the intralobular stroma compartments could be differentiated and classified as being positive or negative for cleaved caspase-3 staining. During this examination, endothelial cells were avoided, and 100 cells in each image were randomly identified and putatively differentiated as being either fibroblasts or immune cells by a single classifier. In addition, differentiated cells were simultaneously classified as being either positively or negatively stained for cleaved caspase-3. This approach resulted in 300 cells being differentiated and these data were used to calculate the percentages for each cell type for each experimental quarter.

Ki-67 stained sections: For the Ki-67 stained sections, 8 mammary parenchyma fields of view were randomly identified and imaged for each experimental quarter tissue section. Regions of mammary parenchyma were identified using DAPI nuclear staining as a morphological index. Images were acquired using a Nuance FX multispectral imaging system (Perkin Elmer, Waltham, MA) affixed to a Nikon Eclipse E600 epifluorescence microscope (Nikon Instruments Inc., Melville, NY) with a Plan Fluor 40x objective. A mercury lamp was used as the excitation light source, and filter cubes, fitted with long pass emission filters, were used to excite specific secondary antibody and DAPI fluorophores. The Nuance system was configured to use tailored emission spectra to visualize DAPI counterstained nuclei and positively stained Ki-67 nuclei. The product of this approach was 2 images for each field of view; 1 image containing DAPI counterstained nuclei and 1 image containing Ki-67 stained nuclei. Correspondingly, a total of 576 images were produced from the 288 imaged fields of view. A total area of 0.718 mm^2 was imaged for each experimental quarter (8 microscopic fields of view) using the configured microscope.

Acquired images were analyzed by counting the number of positively stained Ki-67 nuclei within the mammary epithelial and stromal compartments in each image. The respective compartmental counts completed for each image were summed across the 8 random images acquired from each experimental quarter as described earlier to produce a total number of positive cells across a constant area examined.

4.3.4. Statistical analysis

Cleaved caspase-3 nuclei: The total number of positively stained cleaved caspase-3 nuclei in the epithelial and luminal compartments, quantified across the 6 images per experimental quarter,

were analyzed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). Total epithelial and luminal cleaved caspase-3 cell counts served as the dependent variable in separate models. The fixed independent effects of quarter treatment (n = 2) and day euthanized (n = 2) were included in both models; their interaction was included if $P \leq 0.10$. Cow nested within day of euthanasia was specified as a random effect in both models. The positive cleaved caspase-3 nuclei counts in the luminal compartment were non-normally distributed and yielded non-normally distributed residuals when tested against the model's effects. In response, nuclei counts were log_{10} transformed and analyzed to satisfy the assumptions of normal distribution and equal variance. All presented least squares means were estimated using untransformed nuclei counts, but the P-values produced by the log_{10} transformed luminal nuclei count model are reported.

Differential cleaved caspase-3 intralobular stroma cell counts were also analyzed using the MIXED procedure. Each cell type percentage served as the dependent variable in separate models. Models were identical and included the fixed independent effects of quarter treatment (n = 2) and day euthanized (n = 2) and their interaction if $P \leq 0.10$. Cow nested within day of euthanasia was specified as a random effect in all models. Resultant least squares means were contrasted using Fisher's least significant differences test.

Ki-67 nuclei: The summed number of Ki-67 stained nuclei in the epithelial and stromal compartments were analyzed using the MIXED procedure as the dependent variable in separate models. The models' effects were identical to that used to test cleaved caspase nuclei counts. The positive Ki-67 nuclei counts in the stroma compartment were also non-normally distributed and yielded non-normally distributed residuals when tested against the model's effects; these counts were log_{10} transformed and analyzed as described earlier. Again, the presented least squares means for epithelial and stromal Ki-67 nuclei counts were calculated using untransformed nuclei counts, but *P*-values yielded by the log_{10} transformed stroma nuclei count model are reported.

4.4. Results

4.4.1. Cleaved caspase-3 positive nuclei

Representative images of cleaved caspase-3 staining are presented in Figure 4.1 and the summed cleaved caspase-3 positive nuclei least squares mean counts recorded for the epithelial compartment are summarized and contrasted in Table 4.2. Cleaved caspase-3 nuclei counts in the epithelial compartment tended to be higher in challenge quarters than saline quarters (1.6 vs. 2.6 ±

0.4 nuclei; *P* = 0.060) but nuclei counts were not affected by day of euthanasia (*P* = 0.29). Positive cleaved caspase-3 counts in the epithelial compartment were not influenced by the effect of quarter treatment interacting with euthanasia day (*P* = 0.31).

Summed cleaved caspase-3 positive nuclei counts for the luminal compartment are also presented and contrasted in Table 4.2. The number of cleaved caspase-3 positive nuclei in challenged epithelial lumens was 10-fold greater than that observed in saline quarters (2.0 vs. 0.2 ± 0.5 nuclei; *P* = 0.002). Neither the effect of euthanasia day nor the effect of euthanasia day interacting with quarter treatment affected the number of cleaved caspase-3 positive nuclei in epithelial lumens (*P* = 0.52 and 0.19; respectively).

Overall, a greater percentage of positively stained cleaved caspase-3 nuclei in the intralobular stroma compartment were present in saline quarters than challenge quarters (45.9 vs. 36.9 ± 2.1; *P* < 0.0001); but, the percentage of positively stained cells did not differ between days 5 or 10 of euthanasia (41.1 vs. 41.7 ± 2.8, respectively; *P* = 0.88). The differential intralobular stroma cell type percentages for cells staining positive and negative for cleaved caspase-3 are presented and contrasted in Table 4.3. A greater percentage of positively stained fibroblasts were observed in saline infused quarters than *Staph. aureus* quarters (24.7 vs. 15.7 ± 1.2; *P* < 0.0001). Conversely, saline quarters displayed a lower percentage of immune cells staining positive for cleaved capase-3 than challenge quarters (27.8 vs. 37.0 ± 1.5; *P* < 0.0001). Saline quarters did not differ from *Staph. aureus* challenged quarters in the percentage of fibroblasts not stained for cleaved caspase-3 or the percentage of immune cells positive for cleaved caspase-3 expression (*P* = 0.77 and 0.98; respectively). Furthermore, the effect of euthanasia day and the effect of euthanasia day interacting with treatment did not significantly affect any of the cell type percentages, staining either positive or negative for cleaved capsase-3 (*P* ≥ 0.37).

4.4.2. Ki-67 positive nuclei

Representative images of Ki-67 staining are presented in Figure 4.2, and summed Ki-67 nuclei least squares mean counts for the epithelial compartment are summarized and contrasted in Table 4.4. Total Ki-67 positive nuclei counts in the epithelial compartment of saline infused quarters were not different from those in challenge quarters (*P* = 0.97) and were not different between mammary tissues collected 5 or 10 days post challenge (*P* = 0.78). The effect of quarter treatment

interacting with euthanasia day did not affect positive Ki-67 nuclei counts in the epithelial compartment (P = 0.98).

Summed Ki-67 nuclei least squares mean counts for the intralobular stroma compartment are also summarized and contrasted in Table 4.4. The number of Ki-67 positive nuclei in the intralobular stroma compartment was 2.1-fold higher in challenge quarters than saline quarters (38.1 vs. 18.0 ± 5.6 nuclei; P = 0.020). The effects of euthanasia day and the interaction of euthanasia day and quarter treatment did not influence positive Ki-67 nuclei counts in the intralobular stroma compartment (P = 0.27 and 0.36; respectively).

4.5. Discussion

A chief objective of this study was to examine apoptosis and proliferation of mammary epithelial cells in uninfected and *Staph. aureus* infected mammary glands that were stimulated to rapidly grow and develop using an induced mammary growth protocol. As expected, *Staph. aureus* infected mammary tissues tended to have a greater number of mammary epithelial cells staining positive for the utilized apoptosis marker, cleaved caspase-3, than uninfected quarters. This observation is consistent with that of Long et al. (2001) and Andreotti et al. (2017) who both reported a greater number of mammary epithelial cells undergoing apoptosis in mastitic quarters than uninfected quarters. Differing from these previous reports was the observation that *Staph. aureus* and saline infused quarters had a similar number of proliferating epithelial cells; Long et al. (2001) and Andreotti et al. (2017) both reported a greater number of proliferating epithelial cells in mastitic quarters. This discrepancy is believed to be a function of the different physiological states of mammary tissues being examined. As discussed, the tissues examined here were stimulated to rapidly grow and develop whereas the mammary tissues examined by Long et al. (2001) and Andreotti et al. (2017) were from lactating and actively involuting mammary glands, respectively; limited mammary growth is expected to occur during these physiological states. Others have reviewed (Leoni et al., 2015; Landen et al., 2016) how tissue insults activate wound repair mechanisms which promote cellular proliferation in the affected tissues and drive tissue remodeling and healing. It is suspected that any wound repair mechanisms that would have been activated in the *Staph. aureus* quarters would not have had an additive effect in increasing epithelial proliferation because epithelial proliferation had already been upregulated via the estradiol and progesterone treatment; accordingly, no difference would be observed. Regardless, when these

mammary tissues were examined previously (Enger et al., 2018), less mammary epithelium was observed in challenge quarters, and this observation is associated with the increased epithelial apoptosis described here. Overall, an impairment in the growth of mammary epithelium is indicated.

Another key objective of this evaluation was to characterize the occurrence of cellular proliferation in the intralobular stromal compartment of the saline and *Staph. aureus* infused glands. A greater number of proliferating cells were present in the intralobular stromal compartment of *Staph. aureus* challenge quarters than saline quarters, which was expected, and agrees with previous reports (Long et al., 2001; Andreotti et al., 2017). An increase in the number of proliferating cells in the intralobular stromal compartment would be expected to contribute to the previously observed larger areas of intralobular stroma in *Staph. aureus* infused quarters (Enger et al., 2018). Other investigators have also described increased stromal tissue areas in infected quarters relative to uninfected quarters for both lactating (Spencer, 1949; Akers and Thompson, 1987) and non-lactating glands (Sordillo et al., 1989; Trinidad et al., 1990; Andreotti et al., 2014), and the increased cellular proliferation of cells in the intralobular stromal compartment may, in part, contribute to the reported increased areas of intralobular stroma. Nonetheless, the heterogeneous cell population comprising the intralobular stroma compartment should be considered for an appreciation of the cellular and tissue changes occurring as a result of *Staph. aureus* challenge. This could not be accomplished using the florescent microscopy approach used here since limited cellular morphology is present in florescent imaging and limited cellular morphological features were present to differentiate cells. Regardless, the increased proliferation observed in the intralobular stromal compartment of *Staph. aureus* challenged glands is believed to be resultant of an increase in the number of proliferating fibroblasts, in response to wound repair mechanisms, and an increase in the number of proliferating immune cells, undergoing selection and expansion. These immune cells would be expressing proliferation/survival associated proteins.

Overall, *Staph. aureus* challenged mammary tissues contained a lower percentage of apoptotic cells than saline quarter tissues, which further coincides with the greater areas of intralobular stroma in *Staph. aureus* quarters relative to saline. A novel facet of this investigation was the putative differentiation of intralobular stromal cells staining positive and negative for cleaved caspase-3. It was observed that a greater percentage of non-apoptotic immune cells were

present in *Staph. aureus* quarters than saline quarters. This is not surprising as the increased infiltration of immune cells into the examined mammary tissues would increase the proportion of immune cells in the stroma, which have infiltrated in response to IMI. More interestingly, a greater percentage of non-apoptotic fibroblasts were present in *Staph. aureus* infused quarters than saline quarters. The consequence of fewer apoptotic fibroblasts in the *Staph. aureus* infected glands may contribute to the accumulation of connective tissues in the gland and result in the continued presence of intralobular stromal tissue in these glands. Andreotti et al. (2014) previously described increased amounts of type 1 collagen in actively involuting, chronically infected, *Staph. aureus* quarters relative to uninfected quarters. In the context of the present experiment, Howe et al. (1975) and Croom et al. (1976) examined mammary tissues from non-pregnant, non-lactating animals that were subject to similar hormonal induction protocols. These researchers observed a continued regression of intralobular stroma and expansion of luminal space when the collected mammary tissues were examined throughout the progression of the induction protocol. When these reports are considered with the results of the present study, it indicates that the intralobular stromal compartment is failing to regress or is actively expanding in the *Staph. aureus* challenged glands, which would not allow for the expansion of the luminal space. Either way, an impairment in mammary development is indicated.

Paralleling the result from the first examination of these tissues (Enger et al., 2018), the effect of euthanasia day did not influence any of the measures examined. As previously discussed by Enger et al. (2018), it may be that examining tissues at 5 days post-challenge was too late to capture the temporal changes occurring in the mammary tissues resulting from the *Staph. aureus* challenge.

4.6. Conclusion

The results of this study were that *Staph. aureus* challenge of rapidly growing, non-lactating mammary glands increased the amount of mammary epithelial cells undergoing apoptosis but did not affect the number of proliferating mammary epithelial cells. Furthermore, a greater number of proliferating cells were present in the intralobular stromal compartment of *Staph. aureus* challenged quarters than saline quarters, which was accompanied by a lower percentage of apoptotic cells present in the intralobular stromal compartment of *Staph. aureus* glands. When these results are considered with the results from the previous examination of these tissues (Enger

et al., 2018), it indicates that IMI in rapidly growing non-lactating mammary glands limits gland growth by differentially affecting cellular apoptosis and proliferation in affected tissues, which is expected to impair future milk yield and reduce productive herd life.

4.7. Acknowledgments

Table 4.1. Antibodies, manufacture, catalog numbers, and dilutions employed to stain cleaved caspase-3 and Ki-67 positive nuclei in mammary tissues.

Antibody	Antibody Type	Manufacturer	Catalog Number	Dilution
Cleaved caspase-3	1°	Cell Signaling	9579	1:200
Ki-67	1°	Thermo Fisher Scientific	9106	1:200
Alexa Fluor 594	2°	Thermo Fisher Scientific	A-11037	1:200

Table 4.2. Least squares mean counts of positive cleaved caspase-3 nuclei in mammary epithelial and luminal compartments[1].

	Treatment				Euthanasia Day			
	Saline	Challenge	SEM	*P*-value	Day 5	Day 10	SEM	*P*-value
Epithelium	1.6	2.6	0.4	0.06	1.7	2.5	0.5	0.29
Lumen	0.2	2.0	0.5	< 0.01	1.3	0.8	0.5	0.52

[1]Data expressed as number of positive cells observed across 6 parenchymal tissue fields of view (0.458 mm^2).

Table 4.3. Least squares mean percentages[1] of fibroblast and immune cell types in the intralobular stromal compartment staining positive (+) or negative (-) for cleaved caspase-3.

	Treatment				Euthanasia Day			
	Saline	Challenge	SEM	*P*-value	Day 5	Day 10	SEM	*P*-value
Fibroblast +	24.7	15.7	1.2	< 0.01	20.1	20.3	1.4	0.91
Fibroblast -	26.7	26.4	1.4	0.77	27.4	25.7	1.7	0.51
Immune cell +	21.2	21.2	1.5	0.98	21.0	21.4	2.0	0.90
Immune cell -	27.8	37.0	1.5	< 0.01	32.0	32.8	1.9	0.77

[1]Data expressed as the percentage of cells differentiated from 3 tissue fields of view (100 cells/field of view).

Table 4.4. Least squares mean counts of positive Ki-67 nuclei in mammary epithelial and stromal compartments[1].

	Treatment				Euthanasia Day			
	Saline	Challenge	SEM	*P*-value	Day 5	Day 10	SEM	*P*-value
Epithelium	32.1	32.2	4.7	0.97	33.3	30.9	6.2	0.78
Stroma	18.0	38.1	5.6	0.02	31.9	24.3	5.8	0.27

[1]Data expressed as number of positive cells observed across 8 parenchymal tissue fields of view (0.718 mm^2).
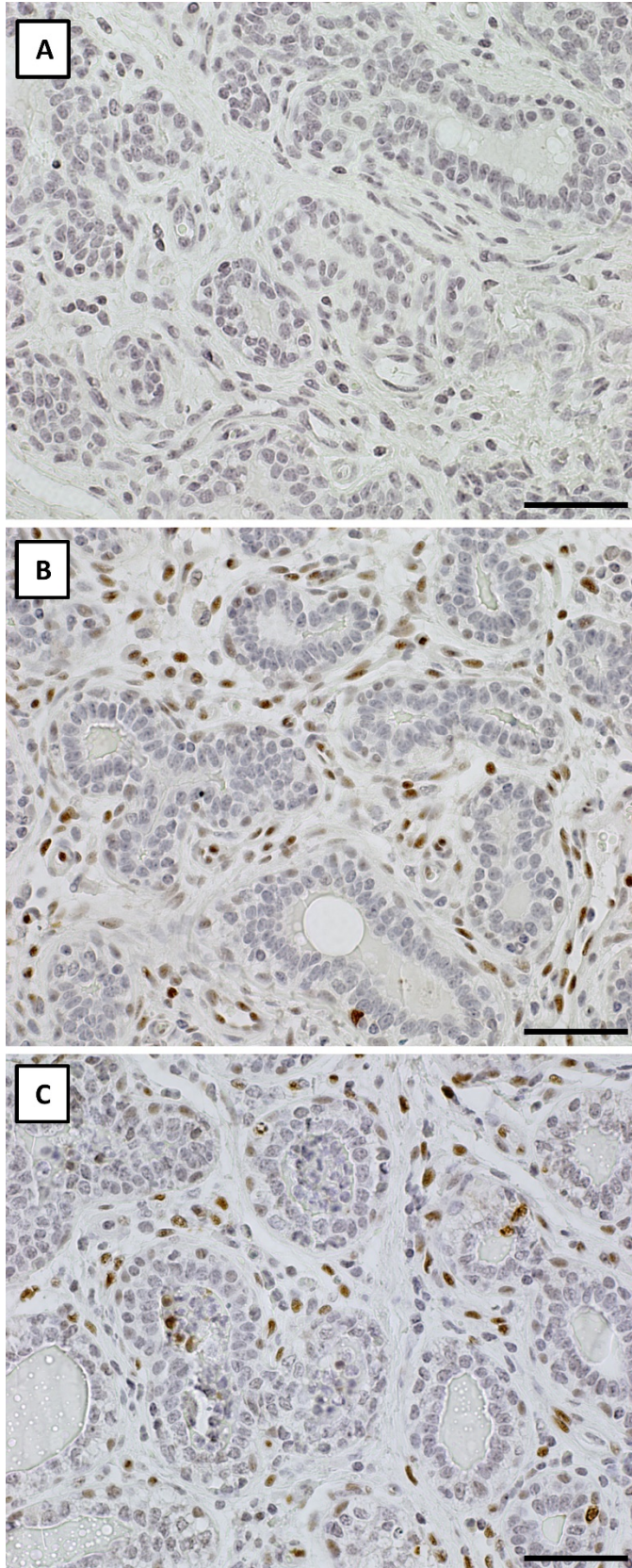
Figure 4.1. Staining of cleaved caspase-3 positive nuclei in mammary tissues from saline and *Staph. aureus* infused glands. Panel A depicts a negative control section where the primary cleaved caspase-3 antibody was omitted; sections were lightly counterstained with hematoxylin. Panel B depicts tissue from a saline infused quarter with extensive staining for cleaved caspase-3 (brown nuclei) for nuclei in the intralobular stroma compartment but limited staining of mammary epithelial nuclei. Panel C displays tissues from a *Staph. aureus* infused gland with several cleaved caspase-3 positive nuclei in the luminal compartment as well as in the mammary epithelium. A reduced percentage of positive cleaved caspase-3 nuclei in the intralobular stroma compartment is also evident. Magnification is the same for all images and scale bars = 50 μm.
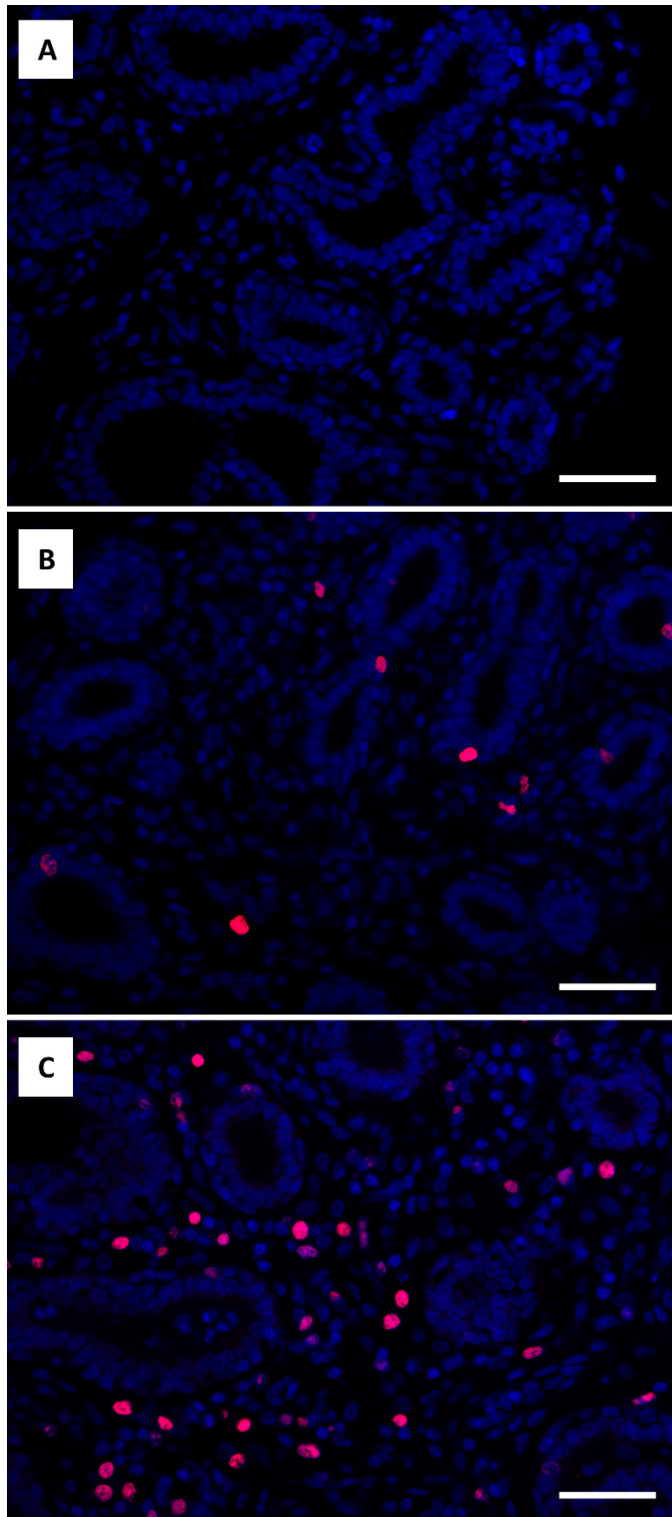
Figure 4.2. Staining of Ki-67 positive nuclei in mammary tissues from saline and *Staph. aureus* infused glands. Panel A depicts a negative control section where the primary antibody (Ki-67) was omitted; nuclei are counter-stained with DAPI (Blue). Panel B depicts tissue from a saline quarter with few intralobular stroma nuclei staining positive for Ki-67 (Red). Panel C portrays mammary tissues from a *Staph. aureus* challenged gland containing a significant number of Ki-67 positive cells in the intralobular stroma compartment. Magnification is the same for all images and scale bars = 50 μm.

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CHAPTER 5: Expression of estrogen receptor α (ESR1) and progesterone receptor (PGR) in rapidly growing, non-lactating mammary glands challenged with *Staphylococcus aureus*

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5.1. Abstract

Intramammary infections (**IMI**) are prevalent in non-lactating dairy cattle and are known to alter mammary structure and negatively affect the amount of mammary epithelium in the gland. Mechanisms responsible for the observed changes in mammary growth during an IMI are poorly understood yet the importance of the key mammogenic hormones driving mammary growth is well recognized. This study's objective was to characterize the expression of estrogen receptor α (**ESR1**) and progesterone receptor (**PGR**) in mammary glands stimulated to grow and develop in the presence or absence of an IMI as well as preliminarily characterize myoepithelial cell's response to IMI. Mammary growth was stimulated in 18 nonpregnant, non-lactating dairy cows, using estradiol and progesterone injections, and 2 culture negative quarters of each cow were subsequently infused with either saline (n = 18) or *Staphylococcus aureus* (n = 18). Mammary parenchyma tissues were collected 5 days (n = 9) or 10 days (n = 9) post-challenge and examined using immunofluorescence microscopy to quantify positive nuclei and characterize staining features. There tended to be a greater number of ESR1 positive nuclei observed across 8 random mammary parenchyma fields of view in saline quarters than *Staph. aureus* challenged (201 vs 163 ± 44 nuclei). Saline quarters also contained a greater number of PGR positive nuclei (520 vs 440 ± 45 nuclei) and myoepithelial cells (971 vs 863 ± 48 nuclei) than challenge quarters. However, when ESR1, PGR, and myoepithelial nuclei counts were adjusted for *Staph. aureus* quarters containing less epithelium, differences between quarter treatments abated. The examined ESR1 and PGR staining characteristics were similar between saline and *Staph. aureus* quarters but were differentially affected by day of tissue collection. Additionally, nuclear staining area of myoepithelial cells was greater in *Staph. aureus* quarters than saline. These results indicate that IMI has little effect on the

number or staining characteristics of ESR1 or PGR positive nuclei, relative to epithelial area, but myoepithelial cells appear to be affected by IMI and the associated inflammation in rapidly growing, non-lactating mammary glands. Accordingly, reductions in mammary epithelium in affected glands are not suspected to be resultant of alterations in the number or staining characteristics of ESR1 or PGR positive mammary epithelial cells.

5.2. Introduction

A primary goal in the rearing and management of production dairy animals is to maximize mammary gland growth and development. This goal is emphasized because milk yield is solely influenced by the number of secretory mammary epithelial cells in the gland and their activity; any improvements or impairments in either of these two factors would ultimately influence milk yields.

It is not surprising that palpable amounts of mammary growth and development occur in concert with gestation. The maturation and growth of the mammary gland during gestation is primarily driven by the pregnancy associated hormones, estradiol and progesterone, and is essential to allow for the development of a gland that is capable of synthesizing and secreting milk to support the neonate. Prior to first gestation, limited amounts of mammary parenchyma are present, and no functional alveolar structures exist that would synthesize and secrete these milk components. To align with the need for the development and maturation of the gland, substantial mammary growth occurs during first pregnancy, which is when the greatest amount of mammary growth and development occurs during an animal's life (Tucker, 1987).

Appreciable amounts of mammary growth also occur in dry cows, during the second half of the dry period, which contributes to the increased milk yields realized in succeeding lactations (Capuco et al., 1997; Capuco and Akers, 1999). Given the considerable amounts of mammary growth that occur during the distinct physiological states of gestation, it is understandable that impairments in mammary growth during this time would have negative impacts on future milk yields.

The chief mechanisms driving mammary growth and morphogenesis have been reviewed numerous times previous (Lyons et al., 1958; Knight and Peaker, 1982; Akers, 1990; Tucker, 2000; Akers, 2017), but it is irrefutably recognized that estradiol and progesterone play central roles in stimulating mammary epithelial cell proliferation (Woodward et al., 1993; Capuco et al., 2002) and promote lobular-alveolar development and glandular morphogenesis (Sud et al., 1968; Howe et al., 1975; Croom et al., 1976). The actions that estradiol and progesterone have in promoting mammary gland growth are largely dependent on these steroid hormones binding with their nuclear receptors, estrogen receptor α (**ESR1**) and progesterone receptor (**PGR**), in mammary epithelial cells, to induce production of mitogenic factors that promote mammary growth and development

(Connor et al., 2007). Changes in the expression of these receptors in mammary epithelium has been associated with changes in mammary growth (Meyer et al., 2006; Tucker et al., 2016b).

Coincident with these periods of rapid mammary growth during pregnancy is the occurrence of bacterial intramammary infections (**IMI**), which are common in primigravid dairy heifers (Trinidad et al., 1990b; Fox, 2009) and dry cows (Eberhart, 1986). Intramammary infections in the mammary glands of non-pregnant heifers (Trinidad et al., 1990a) as well as non-pregnant dry cows (Andreotti et al., 2014) have been shown to induce leukocyte migration, increase stromal tissue areas, and reduce mammary epithelial tissue present in infected glands, demonstrating that IMI negatively impact gland structure in non-lactating glands. However, in these instances, limited amounts of mammary growth and development are expected given that these animals are not pregnant. Yet, previous investigations have reported that infections during first gestation, when considerable amounts of mammary growth are expected, reduce milk yields by approximately 11% (Owens et al., 1991; Oliver et al., 2003). When these observations are taken together, it indicates that IMI during periods of rapid mammary growth can negatively impact the amount of epithelium in the gland, which is expected to reduce future milk yields.

Recently, Enger et al. (2018) stimulated rapid mammary growth and development in glands from non-pregnant dry cows, via controlled injections of estradiol and progesterone at supraphysiological levels, and subsequently challenged these rapidly growing glands with *Staphylococcus aureus* to investigate how IMI, and the resulting immune response, affected the gland. As expected, leukocytosis, increased areas of stromal tissue, and reductions in mammary epithelium resulted; but, it is unknown if IMI reductions in mammary epithelium are influenced by estradiol and progesterone signaling. In this report, we studied these previously collected tissues using immunohistochemistry and quantitative image analysis approaches to investigate whether *Staph. aureus* IMI modulated expression of ESR1 and PGR in rapidly growing mammary tissues. Additionally, a preliminary evaluation of myoepithelial epithelial cells in these tissues was conducted.

5.3. Materials and methods

5.3.1. Study design

The mammary tissues used were collected by Enger et al. (2018). In brief, 19 non-pregnant, non-lactating dairy cows that had been dry for 45 days, were administered daily injections of

estradiol (0.1 mg/kg BW) and progesterone (0.25 mg/kg BW) for 1 week to stimulate mammary growth and development. Three days after the last injection, 2 culture-negative quarters of each cow were infused with either sterile phosphate-buffered physiological saline (n = 19) or *Staph. aureus* (n = 19). All saline infused quarters remained culture negative throughout the trial, and all but one cow developed a *Staph. aureus* intramammary infection in the challenged quarters. At 5 days post-challenge, half the cows were randomly selected and euthanatized for tissue collection; tissues were collected from the remaining animals 10 days post-challenge. Mammary parenchyma, proximal to the teat, but dorsal to the gland cistern was collected from experimental quarters. The mammary tissues collected from the animal that did not establish a *Staph. aureus* IMI were not utilized in the present investigation.

5.3.2. Immunohistochemistry

Two slide sets were prepared to examine ESR1 and PGR staining in mammary epithelial cells from saline and *Staph. aureus* infused mammary quarters. Myoepithelial cells were also identified in both the ESR1 and PGR slide sets, via p40 staining (Parsons et al, 2018), to act as a morphological marker and the myoepithelial cells identified in the PGR slide set were subject to examination. Tissues were sectioned 5 µm thick using a rotary microtome (Model HM 340 E, Microm International GmbH, Waldoff, Germany) and relaxed in a 42°C water bath. For each slide set, 2 serial sections, from each experimental quarter, were mounted on Superfrost™ Plus microscope slides (Thermo Fisher Scientific, Waltham, MA), drained, and dried at 37°C for 24 h. Sections were deparaffinized in 3 changes of a xylene substitute (Clear-RiteTM 3, Thermo Fisher Scientific) and rehydrated to deionized water using a graded ethanol series. Antigens were retrieved by immersing slides in 10-mM citrate buffer, pH 6.0, for 30 min at 95°C. Slides were removed from the heat source after antigen retrieval and allowed to cool to room temperature while remaining submerged in citrate buffer. Slides were subsequently washed in 0.9% NaCl PBS, pH 7.4, and a hydrophobic barrier pen (Liquid Blocker, Daido Sangyo Co., Ltd, Saitama, Japan) was used to separate tissue sections on the same slide to prevent antibody mixtures on different sections from comingling. All sections were subsequently blocked using a universal blocking agent (CAS Block, Thermo Fisher Scientific) for 30 min.

CAS Block was aspirated from the sections, and 100 µL of a primary antibody mixture was applied to a single section on each slide; the remaining section served as the negative control and

was covered with 100 µL of CAS Block. The primary antibody mixture used for the ESR1 slide set included an antibody to detect ESR1 and an antibody to detect p40 (Table 5.1). The primary antibody mixture used for the PGR slide set included an antibody to detect PGR and the p40 antibody (Table 5.1). Antibodies were mixed in CAS Block using the dilutions specified in Table 5.1 and incubated with the respective slide sets in a dark humidified chamber at 4°C for 16 h. After primary antibody incubation, solutions were aspirated, and sections were washed thrice with PBS for 5 min each washing. All tissue sections were then incubated with 100 µL of a secondary antibody mixture at room temperature for 60 min in a dark humidified chamber to detect primary antibodies with the desired fluorophore. Secondary antibodies were also suspended in CAS Block at the dilutions specified in Table 5.1. Secondary antibody mixtures were aspirated and sections were washed thrice more with PBS for 2 min each washing. Residual hydrophobic barrier pen outlines were removed and sections were coverslipped using ProLong™ Gold Antifade Mountant containing DAPI (Thermo Fisher Scientific). The coverslipping mountant was cured overnight before sections were imaged.

5.3.3. Imaging and image analysis

Eight mammary parenchyma fields of view were randomly identified and imaged for each experimental quarter tissue section. Regions of mammary parenchyma were identified using DAPI nuclear staining as a morphological index. Images were acquired using a Nuance FX multispectral imaging system (Perkin Elmer, Waltham, MA) affixed to a Nikon Eclipse E600 epifluorescence microscope (Nikon Instruments Inc., Melville, NY) with a Plan Fluor 40x objective. A mercury lamp was used as the excitation light source and filter cubes, fitted with long pass emission filters, were used to excite specific secondary antibody and DAPI fluorophores. The Nuance system was configured to use multiple, tailored, emission spectra to visualize DAPI counterstained nuclei and positively stained ESR1, PGR, and p40 nuclei. As a result, 3 images for each field of view were produced for each slide set and used for subsequent analysis; one image depicted DAPI counterstained nuclei, one depicted p40 stained nuclei, and the third depicted either ESR1 or PGR positive nuclei, depending on the slide set. Correspondingly, a total of 864 images were produced from the 288 imaged fields of view for each slide set.

Analysis of the acquired images was twofold and included an enumeration of the number of positively stained nuclei and characterization of positively stained nuclei. For each respective slide

set, the number of positively stained ESR1 or PGR nuclei in each field of view were manually counted using a composite image (DAPI + p40 + ERS1 or PGR) while the number of positively stained p40 nuclei identified in the PGR slide set were enumerated by CellProfiler 2.2.0 (Kamentsky et al., 2011). Counts produced for each field of view were summed to produce a total number of positive cells across the 8 imaged fields of view of mammary parenchyma, similar to the approach described previously (Long et al., 2001). With the microscope configuration used, the total tissue area analyzed in 8 microscopic fields was 0.718 mm^2.

CellProfiler 2.2.0 (Kamentsky et al., 2011) was used to analyze morphological and staining characteristics of cell nuclei that were positively stained for ESR1, PGR, and p40. This was achieved by creating two CellProfiler pipelines that were unique to the ESR1 or PGR slide sets. In each pipeline, the three images for each field of view were submitted and designated as being from the same field of view. First, all nuclei in the DAPI counterstained image were identified and defined as areas of interest that could be examined further in companion images of the same field of view to identify positively stained nuclei. This approach was employed to eliminate background signal (cytoplasmic and stromal auto-florescence and/or secondary antibody aggregates) that could be incorrectly classified as positively stained nuclei. Objects identified as positively stained ESR1, PGR, and p40 nuclei were outlined by CellProfiler on the entered images and the resulting images were inspected to confirm that positive nuclei were correctly identified as a quality control measure. In instances where objects were not correctly identified, artifacts were manually effaced, and the image was reanalyzed by CellProfiler. If nuclei were still not correctly identified, the image was removed.

For the ESR1 slide set pipeline, CellProfiler intensity measures (mean intensity and integrated intensity) and area and shape measures (object size and object eccentricity) were applied to all ESR1 identified nuclei objects. These same measures were applied to all PGR positive nuclei objects identified in the PGR slide set. Positively stained p40 nuclei were examined in the PGR slide set only and subject to area and shape measures (object size and object eccentricity). An overall mean for the respective measures was calculated for each experimental quarter using the objects identified across all the analyzed fields of view.

5.3.4. Statistical analysis

The total number of positively stained ESR1 and PGR mammary epithelial cells quantified from the 8 images per experimental quarter were analyzed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). Total positive ESR1 and PGR cell counts served as the dependent variable in separate models. The fixed independent effects of quarter treatment (n = 2) and day euthanized (n = 2) were included in both models; their interaction would be included when $P \le 0.10$. Cow nested within day of euthanasia was specified as a random effect in both models. For the ESR1 data set, positive ESR1 nuclei counts were non-normally distributed and produced non-normally distributed residuals when tested against the model's effects. In response, $\log_{10}$ transformed counts of ESR1 positive nuclei were computed and analyzed to satisfy the assumptions of normal distribution and equal variance. Least squares means were estimated using untransformed positive ESR1 and PGR nuclei counts but P-values produced by the $\log_{10}$ transformed ERS1 model are reported. Least squares means produced by the models were contrasted using Fisher's least significant difference test.

CellProfiler intensity, area, and shape measures for ESR1, PGR, and p40 stained nuclei were analyzed using the MIXED procedure as dependent variables in separate models. The fixed, independent effects of quarter treatment (n = 2) and day euthanized (n = 2) were included in each model; their interaction would be included if $P \le 0.10$. Cow nested within day of euthanasia was specified as random effect in all models. Resultant least square means estimated by the models were contrasted using Fisher's least significant difference test.

5.4. Results

5.4.1. ESR1 positive nuclei counts and staining characteristics

Representative images of ESR1 staining are presented in Figure 5.1 and summed ESR1 positive nuclei least squares mean counts are summarized and contrasted in Table 5.2. Total ESR1 positive nuclei counts in saline infused quarters tended to be higher than those in *Staph. aureus* challenged quarters (201 vs 163 ± 44 nuclei; P = 0.098). Additionally, ESR1 positive nuclei counts were lower in mammary tissues collected 5 days post-infusion than in tissues collected 10 days post-infusion (130 vs. 234 ± 59 nuclei; P = 0.044). Positive ESR1 nuclei counts were not influenced by quarter treatment interacting with euthanasia day (P = 0.51).

A total of 256 imaged fields of view were ultimately analyzed to examine ESR1 stained nuclei characteristics; 7,118 ESR1 stained nuclei were identified from these images and subject to the described CellProfiler measures. The least squares means produced for the selected staining characteristics of positively stained ESR1 nuclei are also presented and contrasted in Table 5.2 for the main effects of quarter treatment and day of euthanasia. In positively stained ESR1 nuclei, stained nuclear area was not found to differ between saline and *Staph. aureus* infused quarters (P = 0.81) and positively stained nuclear area was also not influenced by day of euthanasia (P = 0.56). Eccentricity of ESR1 nuclear staining was not different between positively stained nuclei in saline and challenged quarters (P = 0.32) and was not influenced by day of euthanasia (P = 0.51). Mean intensity of staining in ESR1 positive stained nuclei did not differ between saline and *Staph. aureus* infused glands (P = 0.106) nor did mean integrated intensity of ESR1 positive nuclei (P = 0.30). Neither mean or integrated intensities were influenced by day of euthanasia ($P \geq 0.28$). No interaction was observed between day of euthanasia and quarter treatment on any of the ESR1 nuclear staining characteristics measured ($P \geq 0.24$).

5.4.2. PGR positive nuclei counts and staining characteristics

Representative images of PGR staining are also presented in Figure 5.1 and the summed PGR positive nuclei least squares mean counts are presented and contrasted in Table 5.3. Saline infused quarters contained a greater number of summed PGR positive nuclei counts than challenged quarters (520 vs 440 ± 45 nuclei; P = 0.024); but, positive PGR nuclei counts were not influenced by day of euthanasia (P = 0.78). No interaction was observed between the effects of day of euthanasia and quarter treatment on summed PGR counts (P = 0.93).

A total of 280 imaged fields of view were ultimately utilized to examine PGR stained nuclei characteristics; 19,230 PGR stained nuclei were identified from these images and subject to the described CellProfiler measures. The least squares mean staining characteristics for positively stained PGR nuclei are presented and contrasted in Table 5.3 for the main effects of quarter treatment and day of euthanasia. Nuclear staining area of positively stained PGR nuclei was not different between quarter treatments (P = 0.98), but the area of nuclear staining in PGR stained nuclei was significantly lower in tissues collected 5 days post-challenge than those collected 10 days post-challenge (477 vs 545 ± 19 pixels; P = 0.022). Eccentricity of PGR nuclear staining was not different between saline and *Staph. aureus* infused quarters, but PGR staining in nuclei of tissues

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collected 5 days post-challenge (eccentricity = 0.672) tended (P = 0.056) to be less round than in tissues collected 10 days post-challenge (eccentricity = 0.652). Mean intensity of PGR stained nuclei was not different between saline and *Staph. aureus* infused quarters (P = 0.36) nor in tissues collected 5 or 10 days post-challenge (P = 0.21). The mean integrated intensity of PGR stained nuclei did not differ between quarter treatments (P = 0.72); however, the integrated intensity of PGR stained nuclei tended to be lower in tissues collected 5 days post-challenge than tissues collected 10 days post-challenge (35.9 vs 44.6 ± 3.0; P = 0.055). No interaction was detected between quarter treatment and day of euthanasia in their effect on any of the PGR nuclear staining characteristics measured (P ≥ 0.21).

5.4.3. p40 positive nuclei counts and staining characteristics

All 288 imaged fields of view were utilized to quantify the number of p40 stained nuclei and examine the staining characteristics of stained p40 nuclei; 33,072 p40 stained nuclei were identified from these images. p40 nuclei least squares mean counts and staining characteristics are presented and contrasted in Table 5.4. Total p40 positive nuclei counts in saline infused quarters were greater than those in *Staph. aureus* challenged quarters (971 vs 863 ± 48 nuclei; P = 0.036), but not differ between euthanasia days (P = 0.80), nor were counts affected by quarter treatment interacting with day of euthanasia (P = 0.99). Nuclear staining area of positively stained p40 nuclei was significantly lower in saline quarters than *Staph. aureus* quarters (412 vs 447 ± 10 pixels; P = 0.009). The area of p40 nuclear staining was not different between mammary tissues collected 5 or 10 post-challenge (P = 0.18). Eccentricity of p40 stained nuclei was not affected by the main effects of quarter treatment (P = 0.24) or day of euthanasia (P = 0.94). No interaction was detected between quarter treatment and day of euthanasia in their effect on area or eccentricity of p40 nuclear staining (P ≥ 0.31).

5.5. Discussion

5.5.1. ESR1 positive nuclei counts and staining characteristics

A primary objective of this evaluation was to determine if expression of ERS1 in mammary epithelial cell nuclei was altered by *Staph. aureus* IMI and the immune response initiated to address the IMI in glands that were simulated to rapidly grow and develop. There was a tendency for *Staph. aureus* infused mammary glands to have fewer ESR1 positive nuclei than saline infused glands. This reduction in positive ESR1 nuclei is largely a function of reduced amounts of mammary epithelium

in the *Staph. aureus* infused mammary glands previously reported by Enger et al. (2018). When the ESR1 nuclei counts from these *Staph. aureus* infused glands were adjusted for having less epithelium (a mean reduction of 13%, as reported by Enger et al. (2018)), no difference in the number of ESR1 positive nuclei remained between saline and *Staph. aureus* infused quarters (201 vs 187 ± 47 nuclei, respectively; *P* = 0.44). Accordingly, it is reasonable to expect that the percentage of positive ESR1 mammary epithelial cells does not differ between the saline and *Staph. aureus* infused glands; but, it is recognized that *Staph. aureus* infused quarters would contain a lower absolute number of ESR1 positive mammary epithelial cells than saline glands. Regardless, given that a difference in the percentage of positive nuclei was not indicated from this examination, it suggests that abundance of ESR1 positive nuclei is not contributing to reduced tissue areas of mammary epithelium in *Staph. aureus* challenged glands (Enger et al., 2018).

Coinciding with the lack of differences in the number of ESR1 positive nuclei between treatments, was similar staining characteristics of ESR1 positive nuclei in saline and *Staph. aureus* infused glands. No differences in ESR1 nuclear staining area, eccentricity, or the employed staining intensity measures were observed between treatments. The significance of ESR1 staining intensity has been highlighted in previous works as Geiger et al. (2017) found that increased ESR1 staining intensity was associated with increased levels of mammary growth. Similarly, Tucker et al. (2016b) reported significant reductions in the mammary growth of calves treated with an antiestrogen, ablating estradiol's mammogenic effects. This reduction in mammary growth was associated with substantial reductions in the staining intensity of ESR1 positive mammary epithelial cells. No differences were observed between ESR1 staining characteristics of saline and *Staph. aureus* infused quarters indicating that IMI does not influence ESR1 abundance in mammary epithelial nuclei.

A fewer number of ESR1 positive nuclei were present in mammary tissues collected 5 days post-challenge than 10 days post-challenge. Two factors are expected to be influencing this result. First, all utilized animals in this experiment were synchronized to similar days of estrous before receiving estradiol and progesterone injections, confounding this observation with the effect of estrous day. It is well recognized and documented that expression of ovarian hormone receptors, specifically ESR1 in this instance, is temporal and can be influenced by reproductive status (Schams et al., 2003) and day of estrous (Spencer and Bazer, 1995; Silva et al., 2014). Secondly, because

supraphysiologic dosages of estradiol and progesterone were administered to the utilized animals to stimulate rapid mammary growth, a "knock down" in the number of positive ESR1 nuclei was expected. Geiger et al. (2017) previously observed that continuous administration of estradiol to prepubertal heifers, via an estradiol implant, reduced the percentage of positive ESR1 mammary epithelial cells. Meyer et al. (2006) also observed a lower percentage of ESR1 positive mammary epithelial cells in heifers injected with estradiol, once daily, for three days prior to euthanasia, compared to heifers not receiving exogenous estradiol. In essence, the fewer number of ESR1 positive nuclei in tissues collected 5 days post-challenge, relative to those collected 10 days post-challenge, could simply be an effect of time from the last estradiol injection. Either way, this observation is of limited significance to the investigation at hand. Should this result be examined in the future, it could be argued that synchronization of animals to similar days in estrous should be avoided so that this effect is not confounding; but, as consequence, more inter-animal variation would be expected. A primary goal of this study's design was to limit inter-animal variation given this study's objective.

The lack of differences between tissues collected 5 or 10 days post-challenge for the examined ESR1 staining characteristics indicates that the localization and abundance of ESR1 in positively stained epithelial nuclei, appear to be consistent and not significantly influenced by day of euthanasia.

5.5.2. PGR positive nuclei counts and staining characteristics

Another key objective of this evaluation was to determine if expression of PGR in mammary epithelial cell nuclei, in rapidly growing and developing glands, was affected by *Staph. aureus* IMI and the associated immune response. When the number of PGR positive nuclei from *Staph. aureus* infused glands were adjusted for having less epithelium, no difference in the number of PGR positive nuclei remained between saline and *Staph. aureus* infused quarters (520 vs 505 ± 49 nuclei, respectively; *P* = 0.69). This is similar to the observation that ESR1 positive nuclei counts were not influenced by quarter treatment but the *Staph. aureus* challenged glands would still contain a lower absolute number of PGR positive epithelial cells than uninfected glands. Scant literature exists describing the expression of PGR in bovine mammary glands with which to compare this observation, but it is expected that alterations in the number of PGR positive mammary epithelial cells would affect the morphogenesis and branching of the glandular epithelial network. This is

because PGR knockout in mice fail to develop side branching alveolar structures in response to estradiol and progesterone treatment (Lydon et al., 2000) and when mammary glands from these mice are transplanted into wild type mice and pregnancy is established, a similar failure in lobular alveolar development occurs (Brisken et al., 1998). Regardless, given that the number of PGR positive mammary epithelial cells did not differ when counts were adjusted for epithelial area, no influence from progesterone signaling is expected to contribute to changes in mammary morphogenesis.

The staining characteristic measures applied to positively stained PGR nuclei did not differ between saline and *Staph. aureus* infused glands. This result further substantiates the absence of PGR abundance affecting the reductions in mammary epithelial area and altering tissue morphology.

No difference in the number of PGR positive nuclei was observed between mammary tissues collected 5 and 10 days post challenge, suggesting that the number of PGR positive nuclei is more constant, and less temporal than the expression of ESR1 in mammary epithelial cells. On the other hand, differences were observed between euthanasia day in nuclear stained area, integrated intensity, and eccentricity. These staining characteristic measures are not entirely independent and are influenced by CellProfiler's functionality and the approach used to analyze the assigned images. Positively stained PGR nuclei were identified based on the presence of signal; because these images are grayscale, signal varied in intensity. For this analysis, a minimum intensity threshold was applied to the entire image set during the identification of positively stained nuclei. This was done to prevent misclassification of background signal and accurately identify positive nuclei. As a result, outlining of positive nuclei by CellProfiler was intensity dependent and therefore, if less intense staining was present inside a mammary epithelial cell nucleus, a smaller object would be outlined. Recognizing this effect, 2 intensity measures were employed. Mean intensity depicts the mean signal intensity within a designated area while integrated intensity is calculated by summing each pixel's intensity in the identified object. Thusly, mean intensity is less likely to represent differences in receptor abundance given signal intensity was used as an identification criterion, while integrated intensity evaluates signal intensity in the context of stained area. With this stipulation, the greater area of nuclear staining observed in mammary epithelial cell nuclei at 10 days post-challenge compared to 5 days post-challenge, contributed to the observed differences in integrated

intensities between the different euthanasia days. This indicates that the abundance of PGR receptor was greater in nuclei from tissues collected 10 days post-challenge. Again, the reasons for this increased expression are not entirely understood but likely to be influenced by estrous day and time since last injection as discussed earlier.

5.5.3. p40 positive nuclei counts and staining characteristics

In this preliminary evaluation, the impacts of *Staph. aureus* IMI and the associated immune response were assessed on myoepithelial cells in rapidly growing non-lactating glands. When the number of p40 positive nuclei were adjusted for *Staph. aureus* challenged quarters containing less epithelium, the earlier described differences in number of positive nuclei in saline and *Staph. aureus* quarters abated (974 vs 988 ± 52 nuclei, respectively; $P = 0.80$). Aside from the number of mammary epithelial cells in the gland, the nuclear area of p40 staining was significantly greater in challenged quarters. Reasons for this observation remain elusive but are presumed to be a result of myoepithelial cells responding to the presence of IMI and immune cell recruitment. Myoepithelial cells in the lactating gland are important for expressing milk from the alveolar lumen during milking, but these cells are also associated with gland morphogenesis and establishing epithelial cell polarity as reviewed by Gudjonsson et al. (2005). In the bovine, myoepithelial cells in the glands of ovariectomized heifers are smaller and irregularly shaped relative to cells in glands from intact heifers (Safayi et al., 2012). Tucker et al. (2016a) also described altered myoepithelial cell organization in mammary glands from heifers treated with an antiestrogen and noted that myoepithelial cells were smaller and spaced farther apart relative to untreated heifers. Although the precise mechanisms responsible for the differential staining between saline and *Staph. aureus* quarters are unknown, some hypotheses can be made. First, given myoepithelial cells are located basolaterally to mammary epithelial cells and help separate these secretory cells from the intralobular stroma compartment, we speculate that these cells may be affected by diapedesis of leukocytes across the epithelial cell layer. Additionally, myoepithelial cells are also recognized to contribute to the synthesis and secretion of proteins comprising the basal membrane (Warburton et al., 1982) and may be making select proteins or synthesizing signaling factors in response to the localized inflammation. Lastly, it is unclear if the observed increase in area of p40 nuclear staining reflects an increase in nuclear size or an increased abundance of p40. It is possible that nuclear reorganization, allowing for the differential expression of genes in response to the localized tissue

inflammation, is contributing to the increased nuclear area of myoepithelial cells in *Staph. aureus* challenged glands. Also, p40 has been previously discussed as having roles in directing the stratification of epithelium and a greater abundance observed here may complement previously reported epithelial hyperplasia occurring in *Staph. aureus* infected glands (Trinidad et al., 1990a). Given this observation, future investigations characterizing IMI's impact on mammary tissues should also include examination of myoepithelial cells.

5.6. Conclusion

To the author's knowledge, this investigation is the first of its kind to investigate how IMI, and the ensuing immune response, affect the abundance and staining characteristics of ESR1 and PGR in mammary epithelial cells or how IMI affects myoepithelial cells in rapidly growing bovine mammary glands. The results of this study indicate that *Staph. aureus* IMI has limited effects on the abundance of ESR1 and PGR positive mammary epithelial cells or the staining characteristics of ESR1 or PGR positive mammary epithelial cells, signifying that the previously described mammary growth failures in *Staph. aureus* infected glands are not resultant of ESR1 or PGR expression changes. Myoepithelial cells were affected by *Staph. aureus* IMI as staining area of the utilized marker differed between saline and *Staph. aureus* infused glands, suggesting these cells are involved with the glands response to IMI. The extent and action in which these cells are implicated in the gland's response to IMI is unknown.

5.7. Acknowledgments

Table 5.1. Antibodies, manufacture, catalog numbers, and dilutions employed to stain estrogen receptor α (ESR1), progesterone receptor (PGR), and p40 positive nuclei in mammary tissues.

Antibody	Antibody Type	Manufacturer	Catalog Number	Dilution
ERS1	1°	Santa Cruz Biotechnology	SC-787	1:100
PGR	1°	Santa Cruz Biotechnology	SC-7208	1:200
p40	1°	Abchem	ab172731	1:50
Alexa Fluor 594	2°	Thermo Fisher Scientific	A-21135	1:200
Alexa Fluor 594	2°	Thermo Fisher Scientific	A-11037	1:200
Alexa Fluor 488	2°	Thermo Fisher Scientific	A-21121	1:200

Table 5.2. Least squares mean counts and staining characteristics of estrogen receptor α (ESR1) positive nuclei in saline and *Staph. aureus* infused mammary gland tissues.

	Treatment				Euthanasia Day			
	Saline	Challenge	SEM	*P*-value	Day 5	Day 10	SEM	*P*-value
Nuclei, count[1]	201	163	44	0.10	130	234	59	0.04
Area, pixels[2]	391	393	18	0.81	381	402	25	0.56
Eccentricity	0.681	0.671	0.007	0.32	0.679	0.674	0.007	0.51
Mean intensity, arbitrary unit	0.076	0.072	0.004	0.11	0.070	0.079	0.006	0.28
Integrated intensity, arbitrary unit	31.5	30.1	3.4	0.30	28.5	33.0	4.7	0.51

[1]Data expressed as the number of positive cells observed in 8 parenchymal tissue fields of view.
[2]Mean nuclear area of ESR1 staining in positively stained nuclei.

Table 5.3. Least squares mean counts and staining characteristics of progesterone receptor (PGR) positive nuclei in saline and *Staph. aureus* infused mammary gland tissues.

	Treatment				Euthanasia Day			
	Saline	Challenge	SEM	*P*-value	Day 5	Day 10	SEM	*P*-value
Nuclei, count[1]	520	440	45	0.02	492	468	60	0.78
Area, pixels[2]	511	511	16	0.98	477	545	19	0.02
Eccentricity	0.666	0.658	0.006	0.19	0.672	0.652	0.007	0.06
Mean intensity, arbitrary unit	0.076	0.074	0.003	0.36	0.071	0.078	0.003	0.21
Integrated intensity, arbitrary unit	40.6	39.8	2.4	0.72	35.9	44.6	3.0	0.06

[1]Data expressed as the number of positive cells observed in 8 parenchymal tissue fields of view.
[2]Mean nuclear area of PGR staining in positively stained nuclei.

Table 5.4. Least squares mean counts and staining characteristics of p40 positive nuclei in saline and *Staph. aureus* infused mammary gland tissues.

	Treatment				Euthanasia Day			
	Saline	Challenge	SEM	*P*-value	Day 5	Day 10	SEM	*P*-value
Nuclei, count[1]	974	863	48	0.04	908	929	58	0.80
Area, pixels[2]	412	447	10	0.01	441	417	12	0.18
Eccentricity	0.770	0.766	0.004	0.24	0.768	0.768	0.005	0.94

[1]Data expressed as the number of positive cells observed in 8 parenchymal tissue fields of view.
[2]Mean nuclear area of p40 staining in positively stained nuclei.
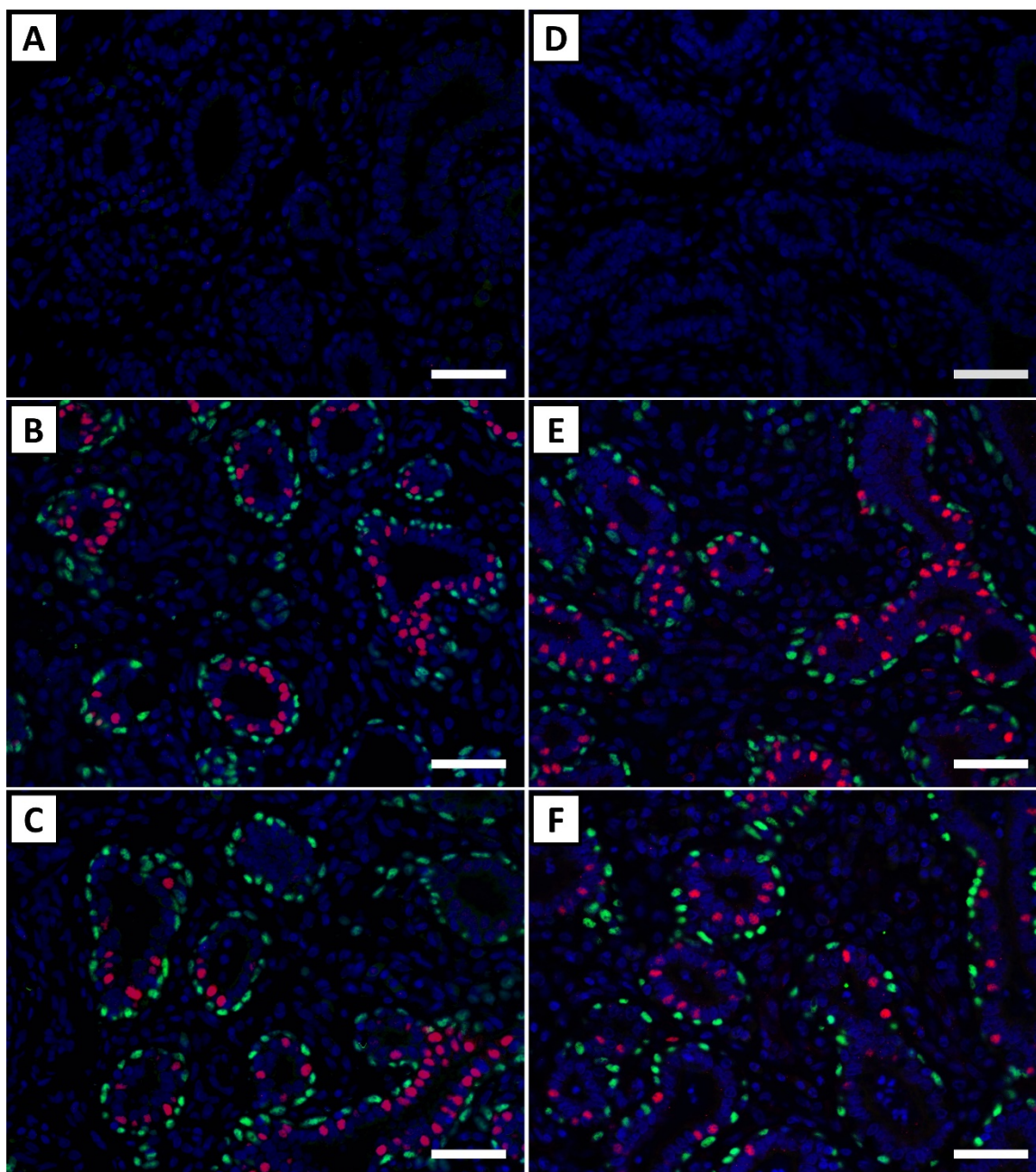
Figure 5.1. Staining of estrogen receptor α (ESR1; Panels A-C), progesterone receptor (PGR; Panels D-F) and p40 (a myoepithelial cell marker; Panels B-C and E-F) in saline and *Staph. aureus* infused mammary gland tissues. Panels A and D depict negative control sections where the primary ESR1 or PGR and p40 antibodies were omitted; nuclei are counterstained with DAPI (Blue). Panels B and E depict ESR1 and PGR (Red) staining in tissues from saline infused glands; myoepithelial cells are labeled by p40 staining (Green). Panels C and F depict ESR1 and PGR staining in *Staph. aureus* tissues along with p40 staining. Magnification is the same for all images and scale bars = 50 μm.

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CHAPTER 6: Conclusions and future directions

The works reported here examined how intramammary infection (**IMI**) affected mammary tissue structure, cellularity, and the expression of key mammogenic hormone receptors in non-lactating mammary glands stimulated to rapidly grow and develop. Intramammary infection elicited an infiltration of immune cells into mammary gland lumens and tissues and also reduced the amount of mammary epithelium, while concurrently increasing the amount of stromal tissue in the gland. A greater number of apoptotic mammary epithelial cells were present in infected quarter tissues. These tissues also contained an increased number of proliferating cells in the stromal compartment, relative to uninfected quarter tissues, while simultaneously having less intralobular stroma cellular apoptosis. These differences in cellular apoptosis and proliferation complement the observed reductions of epithelial area and increased areas of intralobular stroma observed in infected quarters.

An examination of key mammogenic hormone receptors in mammary epithelial cells from infected and uninfected control glands was also undertaken. Neither the abundance of estrogen receptor α (**ESR1**) or progesterone receptor (**PGR**) positive mammary epithelial cells nor the staining intensity of positively stained mammary epithelial cells was significantly influenced by IMI. Myoepithelial cells were also identified and examined. Myoepithelial cells in infected quarters contained a significantly larger area of nuclear staining than myoepithelial cells in uninfected control quarter tissues. The significance of this finding is currently unknown, but it is hypothesized that myoepithelial cells may have a previously unrecognized role in orchestrating/directing the mammary gland's responses to IMI. Potentially, myoepithelial cells could be influencing basal membrane integrity which would be important for maintaining the milk blood barrier or they could be directing leukocyte diapedesis into gland lumens. Future research should validate the observations reported here and consider potential roles that myoepithelial cells have in mastitis resolution in both lactating and non-lactating mammary glands.

Much is still unknown about how IMI affects the growth and morphogenesis of the virgin heifer mammary gland. This study measured the acute, mechanistic response of rapidly growing mammary glands to IMI, but questions still remain about how IMI would affect the long-term growth and morphogenesis of rapidly growing and developing mammary glands. For instance, it is unknown if the observed changes in tissue structure reported here are more localized to tissues

near the gland cistern or if the changes observed here reflect global parenchyma changes. This study could have investigated this question, but the relative distance between the distal parenchyma and gland cistern in dry cows is considerably different from that of primigravid heifer glands, undermining the validity of using this model to investigate this question. To better answer this question, it is recommended that nulligravid heifers or primigravid heifers be utilized. Use of nulliparous or primiparous heifers is also recommended for future works that would build upon this investigation as the cows used are not ideal to understand how IMI influences parenchymal expansion into the mammary fat pad, given that cows would have already experienced this foundational morphogenesis. Regardless of these limitations, a clearer understanding was gained of how IMI affects mammary tissue structure, cellularity, and ESR1 and PGR expression when glands are being stimulated to grow.

The prevalence and impacts of IMI in primigravid heifers affecting future milk production and herd life is well appreciated, but still remains a significant and relatively unaddressed problem. Continued research in both characterizing how the gland is affected by these infections and preventing these infections from occurring will be instrumental in identifying therapeutics for affected glands as well as reducing the prevalence and incidence of this disease. Improvements in each will reduce this disease's impact on the mammary gland itself and the financial burden to the dairy farmer.