Histological tissue structure alterations resulting from Staphylococcus aureus intramammary infection in heifer mammary glands hormonally induced to rapidly grow and develop

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ABSTRACT

Intramammary infections (IMI) are common in nonlactating dairy cattle and are expected to impair mammary growth and development and reduce future milk production. The objective of this study was to histologically evaluate how IMI alter tissue structure in growing and developing heifer mammary glands. A total of 18 nonpregnant, nonlactating heifers between 11 and 14 mo of age were used in the present study. Heifers received daily supraphysiological injections of estradiol and progesterone for 14 d to stimulate rapid mammary growth and development. One-quarter of each heifer was subsequently infused with Staphylococcus aureus (CHALL) while a second quarter served as an uninfected control (UNINF). Heifers were randomly selected and euthanized either the last day of hormonal injections to observe IMI effects on mammary gland growth (GRO), or 13 d post-injections, to observe IMI effects on mammary development (DEV). Mammary tissues were collected from the center and edge parenchymal regions of each mammary gland for morphometric tissue area evaluation. For GRO tissues, CHALL quarters had less epithelial tissue area and marginally more intralobular stroma tissue area than UNINF quarters. Tissue areas occupied by luminal space, extralobular stroma, adipose, and lobular tissue were similar. For DEV tissues, CHALL quarters had less epithelial tissue area and marginally more intralobular stroma tissue area than UNINF quarters. Tissue areas occupied by luminal space, extralobular stroma, adipose, and lobular tissue were similar. For DEV tissues, area occupied by epithelium, luminal space, intralobular stroma, and extralobular stroma did not differ between quarter treatments, but UNINF quarters had more adipose tissue area and marginally less lobular area than CHALL quarters. Results indicate that IMI in growing and developing mammary glands reduces mammary epithelial growth and alters mammary gland development by impairing epithelial branching into the mammary fat pad. Taken together, these tissue changes before calving may have adverse effects on milk production. Therefore, an important focus should be placed on improving udder health in replacement heifers through management strategies that mitigate the deleterious effects of IMI and promote the positive development of the mammary gland.

Key words: mastitis, heifer mastitis, mammogenesis, fat pad

INTRODUCTION

Bovine mastitis is primarily the result of a bacterial IMI and imposes considerable economic losses to US and global dairy industries; costs arise from reduced milk production, discard of abnormal and antibiotic laden milk, premature culling of affected animals, and increased veterinary expenses. Although seldom visibly apparent, nonlactating dairy heifers can also experience IMI before first calving. Heifer IMI prevalence at the quarter level has been documented to range from a low of 26% (Larsen et al., 2021) to a high of 75% (Trinidad et al., 1990b) in intensive management systems in North America.

Intramammary infections in nonlactating mammary glands are a cause for concern, given most postnatal mammary gland growth and development occurs during first pregnancy—also the time of alveoli formation (Swanson and Poffenbarger, 1979). Mammary gland growth (i.e., the accumulation of mammary epithelial cells) is exponential during first pregnancy. The branching epithelial network that is present at the time of breeding is rudimentary and grows into the mammary fat pad, yielding a highly organized network of ductal and secretory epithelial cells by parturition (Tucker, 1987). Concerningly, paralleled with the marked mammary growth and development occurring during the last trimester of gestation is the increased risk for IMI (Trinidad et al., 1990b; Fox et al., 1995). Impairments in mammary gland growth and development during first gestation can shortchange future mammary gland growth.
maturation and reduce the number or functional capacity of secretory epithelial cells. Indeed, prepartum IMI has been reported to elevate SCC and reduce first lactation milk yield (Hallberg et al., 1995; De Vliegher et al., 2005b) and increase culling risk in primiparous cows (De Vliegher et al., 2004, 2005a).

Previous works have reported that IMI negatively affects the nonlactating mammary glands of unbred heifers by reducing epithelial tissue area and increasing mammary connective tissue area (Trinidad et al., 1990a). A recent investigation of Staphylococcus aureus infected dry cow mammary glands noted similar observations; Staphylococcus aureus infected mammary glands that were hormonally stimulated to grow exhibited reduced mammary epithelial tissue area and greater stromal tissue area relative to uninfected mammary glands (Enger et al., 2018). Further evaluation of the same dry cow mammary tissues noted that Staph. aureus IMI increased the number of mammary epithelial cells undergoing apoptosis and increased the number of proliferating stromal cells relative to uninfected mammary glands (Enger et al., 2019). The structural (Enger et al., 2018) and cellular (Enger et al., 2019) changes observed in Staph. aureus infected dry cow mammary glands were associated with an increased abundance of collagen in the infected mammary glands (Enger et al., 2020). When collectively considered, these tissue and cellular changes are expected to reduce future milk production. Despite this, no study has investigated the effect of IMI in rapidly growing and developing heifer mammary glands.

Mammary growth and development during pregnancy are achieved by a complex mixture of hormones and growth factors (Akers, 2006). Chiefly central are the steroid hormones estradiol and progesterone, whose synergized actions are pivotal in mammary morphogenesis (Tucker, 2000). Estradiol is a potent mammary mitogen and stimulates mammary epithelial cell proliferation and ductal lengthening (Woodward et al., 1993; Capuco et al., 2002), while progesterone promotes alveolar and lobular development (Sud et al., 1968; Howe et al., 1975; Croom et al., 1976). Given the undeniable roles of estradiol and progesterone, supraphysiological administration of these hormones has been used in model settings to study mammary growth, development, and lactogenesis (Howe et al., 1975; Croom et al., 1976; Enger et al., 2018). This approach can be more pragmatic for mechanistic examinations than long-term trials, as it provides the ability to directly investigate acute mechanistic responses in condensed timeframes (i.e., weeks not months). The objective of this study was to exploit these mechanisms and acutely stimulate rapid mammary growth and development via supraphysiological administration of estradiol and progesterone to investigate how Staph. aureus IMI, and the associated inflammatory response, alters mammary gland growth and development in the nulligravid heifer mammary gland. We hypothesized that Staph. aureus IMI during rapid mammary growth and development will decrease the tissue areas occupied by mammary epithelium while increasing stromal tissue areas relative to uninfected mammary glands.

**MATERIALS AND METHODS**

This work was approved by The Ohio State University Institutional Animal Care and Use Committee (Protocol 2020A00000024).

**Heifer Selection and Study Design**

A total of 18 nulligravid Holstein dairy heifers between 11 and 14 mo of age were purchased from a single Ohio commercial dairy in 2 cohorts (cohort A, n = 8; cohort B, n = 10) and used in the present study. Enrolled heifers had not been previously inseminated and were identified for study inclusion based on visual observations and examination of the udder and teats. Heifers with udders displaying signs of inflammation (e.g., redness, swelling) or had teats that were flat or inverted were excluded from the study. Heifers exhibiting other clinical signs of disease were also excluded. Selected heifers were purchased and moved to the Ohio Agricultural Research and Development Beef Research Center 1 d before the start of the experiment. At enrollment, the mean age and weight of heifers in cohort A were 382 d (SD = 7) and 320 kg (SD = 28), and the average age and weight for heifers in cohort B were 364 d (SD = 28) and 338 kg (SD = 46). Heifers were housed individually and fed ad libitum for the trial’s duration.

Heifers used were subject to a 27-d experimental timeline depicted in Figure 1. All heifers received daily subcutaneous injections of estradiol and progesterone on d 1 through d 14 of the experiment to stimulate rapid mammary growth and development. One-quarter of each heifer was randomly assigned using a random number generator to be infused with Staph. aureus (CHALL, n = 18) on d 8, while another quarter of each heifer was designated to serve as an uninfected control (UNINF, n = 18). Infusing Staph. aureus 8 d after the first hormone injection allowed for the assessment of how a Staph. aureus IMI affects mammary gland growth in glands that are already experiencing marked degrees of growth, and growth-related processes. Heifers in cohort A did not produce mammary secretions available for sampling before Staph. aureus infusion.
Accordingly, the experimental quarters of each animal were randomly selected without knowledge of current infection status. Secretions samples could, however, be obtained on d 12, 14, 25, and 27 of the trial, and they were used to confirm whether CHALL quarters were infected with *Staph. aureus* throughout the trial. Mammary secretion samples were obtained as previously described by Enger et al. (2018). Samples were also used to randomly assign a culture negative quarter as the uninfected control before tissue collection and to determine whether UNINF quarters remained uninfected until tissue collection day. Heifers in cohort B did produce mammary secretions at the time of treatment allocation, and secretion samples were obtained 2 d before *Staph. aureus* infusion (d 6) to identify culture negative quarters within the udder. As in cohort A, 2 culture negative quarters from each heifer in cohort B were randomly designated as either the CHALL or UNINF quarter. Secretion samples were also collected again as in cohort A on d 12, 14, 25, and 27 of the trial to confirm infection status of experimental quarters.

Half the heifers within each cohort were randomly selected for euthanasia on either the last day of the hormonal injections (d 14; n = 9), or 13 d after the last day of hormonal injections (d 27; n = 9) for mammary tissue collection. Heifer mammary tissues collected on the last day of estradiol and progesterone injections will henceforth be denoted as GRO tissues because the concentrations of estradiol and progesterone levels were highest at this time and would influence the growth of the mammary gland through cellular proliferation (Sud et al., 1968). Mammary tissues collected 13 d after the conclusion of the induction protocol were labeled as DEV tissues because the mammary gland shifts from a nonsecretory state to secretory after estradiol and progesterone administration is ceased (Smith and Schanbacher, 1973, 1974; Howe et al., 1975). Throughout the trial, a commercial iodine teat disinfectant (TEAT-KOTE 10/III, GEA Technologies) was applied daily to reduce likelihood against quarters developing new IMI.

**Estradiol and Progesterone Injections**

The estradiol and progesterone injectables were prepared as detailed by Enger et al. (2018). Briefly, 17β-estradiol (Sigma-Aldrich Co.) and progesterone (Sigma-Aldrich Co.) were dissolved in a 100% ethanol and benzyl benzoate solution and filter-sterilized using a 0.45-µm filter. The filtrate was mixed with autoclaved corn oil yielding a final injectable solution of 10% ethanol, 20% benzyl benzoate, and 70% corn oil by volume. The daily estradiol and progesterone injections were administered subcutaneously on alternating sides of the neck at concentrations of 0.1 and 0.25 mg/kg BW, respectively.

**Intramammary Challenge**

The *Staph. aureus* novel strain isolated by Smith et al. (1998) was prepared and used as the challenge pathogen as previously detailed by Enger et al. (2018). The *Staph. aureus* novel strain was chosen because of its ability to establish an IMI in nonlactating mammary glands that could be maintained throughout the experimental timeframe (Enger et al., 2018) and its ability to induce cell apoptosis of bovine mammary epithelial cells in vitro (Bayles et al., 1998). Briefly, 50-mL of trypticase soy broth was inoculated with a single colony of *Staph. aureus* novel and incubated in an orbital shaking incubator for 6 h at 250 rpm at 37°C. The bacteria were then washed thrice with sterile PBS and adjusted to a concentration of 5 × 10⁸ cfu/mL by measurement of optical density at 600 nm. The
resulting *Staph. aureus* novel suspension was serially diluted with sterile PBS to a target concentration of 5,000 cfu/mL as previously performed by Enger et al. (2018). Tuberculin syringes were loaded with 1 mL of the diluted suspension and transported to the farm on ice to be infused into CHALL quarters using a sterile blunt plastic cannula (Becton, Dickinson and Company) using partial insertion and aseptic procedures detailed previously (Enger et al., 2018). The final dose of *Staph. aureus* novel colony-forming units infused into each mammary gland was 7,000 and 7,900 cfu/mL for cohort A and B, respectively.

**Mammary Secretion Collection and Culture**

Mammary secretion samples were obtained from heifers 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), which remained on teat skin for at least 30 s before removal with a single-use paper towel. Teat ends were scrubbed with 10 × 10 cm² cotton pads soaked in 70% ethanol before mammary secretions were aseptically collected in sterile 5-mL round-bottom polystyrene tubes. Secretions were placed on ice and transported to the Mastitis and Mammary Physiology Laboratory (Wooster, OH) for bacteriological culture. Secretion samples were vortexed and 10 µL of the sample was plated onto esculin blood agar. Cultures were incubated at 37°C and examined at 24 and 48 h after incubation. Samples were considered culture positive if 3 or more colonies of similar morphology were observed. Isolates were confirmed as *Staph. aureus* via coagulase test.

**Euthanasia, Tissue Collection, and Processing**

Heifers were stunned by captive bolt and euthanized via exsanguination. Whole udders were removed, labeled for orientation, and immediately transported to the Mastitis and Mammary Physiology Laboratory for dissection. The udder was placed on a cutting board with teats facing upwards for dissection. Mammary parenchymal tissues were collected from the designated UNINF quarter and CHALL quarter from 2 specific glandular regions: parenchyma that abuts the mammary fat pad, distal to the teat end (edge parenchyma) and mammary parenchyma located slightly above the gland cistern, proximal to the teat end (center parenchyma). Collected mammary tissues were fixed in 10% formalin for 48 h and subsequently transferred and stored in 70% ethanol. Formalin fixed tissues were then grossed and embedded in paraffin.

**Tissue Histologic Analysis**

**Immune Cell Infiltration and Vacuole Scoring.** A hematoxylin and eosin slide set was prepared to evaluate the degree of immune cell infiltration within the luminal space and intralobular stroma, and to assess the degree of vacuolation present in mammary epithelium. Briefly, tissues were sectioned at 4-µm thick using a rotary microtome (model HM 340 E; Microm International GmbH), relaxed in a 42°C water bath, mounted to Superfrost Plus microscope slides (Thermo Fisher Scientific), and dried on a slide warmer. Dried sections were then stained with hematoxylin and eosin as described by Tucker et al. (2016) with modifications (Enger et al., 2018). Hematoxylin and eosin-stained sections were visualized and magnified with an Olympus BX43 microscope (Olympus Corp.) fitted with a SC180 camera (Olympus Corp.). One tissue section was examined for each unique heifer, quarter, and parenchymal region combination; 3 randomly selected fields of view were magnified at 40× using a 4× PlanN objective for a total of 192 images. The acquired 40× images were scored using a graded scoring scale (Figure 2). The individual scorer was blinded to treatments. Luminal infiltration scores ranged from 1 to 3; a score of 1 represented no indication of immune cells within the luminal spaces, a score of 2 reflected one-half of the luminal spaces containing immune cells, and a score of 3 represented over two-thirds of the luminal spaces containing immune cells. Intralobular stroma infiltration scores ranged from 1 to 3; a score of 1 reflected tissue with no evidence of immune cell infiltration, a score of 2 reflected one-half of the intralobular stroma area containing immune cells, and a score of 3 represented over two-thirds of the intralobular stroma area containing immune cells, and a score of 3 reflected tissue with more than two-thirds invaded by immune cells. Vacuolation scores also ranged from 1 to 3; a score of 1 represented mammary epithelium devoid of vacuoles, a score of 2 reflected numerous small vacuoles and few large vacuoles within the mammary epithelium, and a score of 3 represented mammary epithelium with a large accumulation of vacuoles throughout the mammary tissues.

**Quantification of Mammary Tissue Areas.** A second examination was conducted to quantify the amount of relative mammary tissue area occupied by: luminal space, epithelium, lobular tissue, intralobular stroma, extralobular stroma, and adipose tissue. In addition to the 3 randomly selected fields of view magnified at 40× using a 4× PlanN objective, 8 fields of view were randomly magnified at 100× using a 10× PlanN objective. The total number of 40× images was 192 and the total number of 100× images was 512.
and the summed total areas magnified for each tissue section of the 3 and 8 images were 13.0 and 5.7 mm², respectively. Mammary tissue structures were manually traced using CellSens imaging software (Olympus Corp.) to yield the area of each traced object. Extralobular stroma, adipose, and lobule tissue areas were enumerated using 40× images (Figure 3a) while luminal space, epithelium, and intralobular stroma tissue areas were quantified using the 100× images (Figure 3b). Tissue areas are expressed as the area occupied (mm²) per 1 mm² of evaluated mammary tissue.

**Statistical Analysis**

**Immune Cell Infiltration and Vacuole Scoring.** Immune cell infiltration within the intralobular stroma and luminal space and vacuolation of the epithelium was analyzed using PROC MIXED in SAS 9.4 (SAS Institute Inc.). Assigned scores served as dependent variables in 6 separate models where GRO and DEV tissue measures were analyzed separately, given the stark physiological difference in biology of these mammary glands. The final models included the fixed effects of quarter treatment (n = 2) and parenchyma region.
(n = 2) with heifer nested within cohort as a random effect. The interactive term of quarter treatment and region was not reported due to nonsignificance (P ≥ 0.47). Data for each respective model were normally distributed, and all models satisfied the assumptions of equal variance.

**Tissue Areas.** Tissue area measurements were analyzed using PROC MIXED in SAS 9.4. The percentages of area occupied by adipose, extralobular stroma, intralobular stroma, lobule area, epithelium, and luminal space served as dependent variables in separate models (n = 6). The GRO and DEV tissues were analyzed separately once more. The final models included the fixed effects of quarter treatment (n = 2) and parenchyma region (n = 2); heifers nested within cohort were included as a random effect. The quarter treatment by region interaction was not reported due to nonsignificance (P ≥ 0.13). Data for each respective model were normally distributed, and all models satisfied the assumptions of equal variance.

**RESULTS**

**Success of Intramammary Challenge and Hormone Induction Protocol**

After completion of the trial, 2 heifers were excluded from subsequent tissue analyses because the CHALL quarter of 1 heifer spontaneously cured by the time of tissue collection, and the CHALL quarter of another heifer developed a dual infection. Removal of these heifers resulted in 6 animals being present in cohort A (n = 3 GRO heifers and n = 3 DEV heifers) and 10 heifers in cohort B (n = 5 GRO heifers and n = 5 DEV heifers). For the heifers used, all *Staph. aureus* IMI persisted until tissue collection at either d 14 or 27, and all UNINF quarters remained uninfected throughout the trial. The CHALL quarters did not display signs of clinical mastitis throughout the trial.

Mammary gland changes from the 14-d hormone induction protocol were visually and palpably apparent between the GRO and DEV groups at the time of tissue collection (Figure 4). Pronounced fluid accumulation within the udder of the experimental animals occurred 5–8 d after the last estradiol and progesterone injection. The degree of udder engorgement was variable between heifers, as was the onset of the udder becoming distended and engorged with fluid.

**GRO Tissues**

**Immune Cell Infiltration and Vacuole Scoring.** The CHALL quarters exhibited greater immune cell infiltration in the luminal (P = 0.005) and intralobular stromal (P = 0.01) compartments compared with UNINF quarters (Figure 5); the degree of epithelial vacuolation did not differ between quarter treatments (P = 0.30). Neither immune cell infiltration scores, nor epithelial vacuolation scores, differed between center and edge parenchymal regions (P ≥ 0.39 and P = 0.78, respectively).

**Tissue Area Measures.** Mammary tissue area measures for GRO heifers are presented in Table 1. Challenged quarters had less epithelial tissue area (P
= 0.04) and marginally more intralobular stroma tissue area \( (P = 0.06) \) than UNINF quarters. Tissue areas occupied by luminal space, extralobular stroma, adipose, and lobular tissue were not different between UNINF and CHALL quarters \( (P \geq 0.32) \). Center parenchyma had a greater tissue area occupied by epithelium \( (P = 0.01) \), intralobular stroma \( (P = 0.001) \), extralobular stroma \( (P = 0.002) \), and lobular tissue area \( (P < 0.001) \) than edge parenchyma. Edge parenchyma had more adipose tissue area than center parenchyma \( (P < 0.001) \). Luminal space was similar between edge and center parenchymal tissues \( (P = 0.36) \).

**DEV Tissues**

*Immune Cell Infiltration and Vacuole Scoring.* The CHALL quarter tissues exhibited greater immune cell infiltration in both luminal \( (P < 0.001) \) and intralobular stroma \( (P < 0.001) \) compartments than UNINF quarters (Figure 6); the degree of epithelial vacuolation did not differ between quarter treatments \( (P = 0.84) \) but was greater in edge parenchyma than that of center parenchyma tissue regions \( (P < 0.001) \). Luminal and intralobular stroma immune cell infiltration scores did not differ between parenchyma regions \( (P = 0.14 \) and 0.25, respectively).

*Tissue Area Measures.* Mammary tissue measures for DEV heifers are presented in Table 2. Tissue areas occupied by epithelium, luminal space, intralobular stroma, and extralobular stroma did not differ between UNINF and CHALL quarters \( (P \geq 0.30) \). Uninfected quarters had more adipose tissue area than CHALL quarters \( (P = 0.01) \) and marginally less lobular area \( (P = 0.08) \). Edge parenchyma had more adipose than center parenchymal tissue \( (P < 0.004) \) and marginally...
less intralobular stroma \( (P = 0.06) \), but no other tissue area differences were observed \( (P \geq 0.17) \).

**DISCUSSION**

**Evaluation of Model Success**

The goal of this experimental design was to rapidly induce mammary growth and development so that the effects of a sustained *Staph. aureus* IMI on mammary gland growth and development could be separately examined. The greater degree of immune cell infiltration in CHALL quarters indicates that an inflammatory response was initiated to address the *Staph. aureus* IMI. This was expected, as IMI elicits the recruitment of immune cells from the blood to the source of infection (Harmon, 1994). Similar observations have been reported for nonlactating mammary glands, where greater abundance of immune cell infiltration was evident in infected mammary tissues (Boddie et al., 1987; Trinidad et al., 1990a; Enger et al., 2018) and secretions (Sordillo et al., 1987; Hallberg et al., 1995; Watanabe et al., 2017) relative to uninfected quarters.

The 14-d estradiol and progesterone induction protocol used in the present study was chosen to allow for comparison of mammary glands either rapidly growing (i.e., GRO) or developing (i.e., DEV) in response to IMI. These 2 hormones play chief roles in mammary epithelial cell proliferation (Woodward et al., 1993; Capuco et al., 2002) and glandular morphogenesis (Sud et al., 1968; Howe et al., 1975; Croom et al., 1976). Previous literature has reported a lack of significant glandular development and precocious milk secretion in heifers during the immediate hormonal administration period (Sud et al., 1968; Smith and Schanbacher, 1973, 1974), but marked histological development and the onset of secretory activity after the cessation of estrogen and progesterone treatments (Hower et al., 1975; Smith and Schanbacher, 1973, 1974; Delouis et al., 1978). These observations suggest that the onset of udder development (i.e., marked tissue rearrangement and development of secretory capacity) is most associated with the termination of hormone injections rather than during hormone injections. Thus, in our present study, collecting mammary tissues from animals the last day of hormonal injections would be representative of mammary growth, whereas mammary tissues collected 13 d postinjections would be more indicative of mammary development. Mammary development was apparent by increased production of mammary secretions and distention of mammary glands following the cessation of hormones (Figure 4).

**Figure 6.** Mean immune cell infiltration scores and vacuole scoring for 8 uninfected (UNINF) and 8 *Staphylococcus aureus* challenged (CHALL) mammary glands with tissue collection performed 13 d posthormonal injections (i.e., DEV tissues). Each of the previously acquired 40× images were scored on a scale of 1 to 3 by a scorer blinded to treatments. Error bars represent the SE of the respective mean immune cell infiltration and vacuole scores. Asterisks denote differences between CHALL and UNINF quarters within luminal and intralobular stromal areas. ***\( P \leq 0.0001 \).**

**Table 1.** Tissue area measures\(^1\) for mammary tissues collected from 8 uninfected (UNINF) and 8 *Staphylococcus aureus* challenged (CHALL) mammary glands on the last day of estradiol and progesterone injections (i.e., GRO heifers), expressed as the area occupied (mm\(^2\)) per 1 mm\(^2\) of mammary tissue

<table>
<thead>
<tr>
<th>Tissue measure</th>
<th>Treatment</th>
<th>Region</th>
<th>P-value</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UNINF</td>
<td>CHALL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelium(^2)</td>
<td>0.23</td>
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<td>Luminal space(^2)</td>
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<td>0.07</td>
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<td>Intralobular stroma(^2)</td>
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<tr>
<td>Extralobular stroma(^3)</td>
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<td>0.25</td>
<td>0.01</td>
<td>0.55</td>
</tr>
<tr>
<td>Adipose(^3)</td>
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<td>0.28</td>
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<td>0.63</td>
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<td>Lobular area(^3)</td>
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<td>0.47</td>
<td>0.02</td>
<td>&lt;0.001</td>
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\(^1\)The combined tissue areas do not add to 1 mm\(^2\) because different tissue areas were measured using different image sets.

\(^2\)Tissue area measurements enumerated using images magnified at 100× using a 10× PlanN objective.

\(^3\)Tissue area measurements enumerated using images magnified at 40× using a 4× PlanN objective.
Evaluation of GRO Mammary Tissues

The chief aim in generating and examining the GRO mammary tissues was to determine how mammary tissue structures are affected by IMI during acute stimulation of mammary gland growth. The approximate 13% reduction in epithelial tissue area in the CHALL mammary tissues suggests that the presence of *Staph. aureus* IMI reduced epithelial growth, aligning with previous reports (Trinidad et al., 1990a; Enger et al., 2018). Reduced epithelial growth would result from either reduced accretion of mammary epithelial cells, via lesser proliferation or greater apoptosis, or reduced mammary epithelial cell size. It cannot be concluded which contributed to the observations here, but the reduced epithelial tissue areas in infected quarters noted in our current study and other studies (Trinidad et al., 1990a; Enger et al., 2018) are consistently associated with increased intralobular stroma tissue area. Whether impaired mammary growth is resultant of intralobular stroma failing to regress or from the lack of mammary epithelial expansion in the infected quarters, is unknown. However, the negative effect of infection on mammary growth is clear.

The lack of additional tissue area difference between UNINF and CHALL quarter treatments is notable, but not entirely surprising. We posit the lack of further tissue area differences to be resultant of tissues not undergoing pronounced tissue rearrangement (i.e., stromal regression and luminal expansion). Marked increases in luminal space do not occur until the mammary gland shifts toward a pseudolactogenic state, which begins around the last half of gestation (Akers, 2002, 2006). In the case of this induction model, lactogenic capacity is recognized as beginning after the termination of estradiol and progesterone injections (Smith and Schanbacher, 1973, 1974; Howe et al., 1975), and marked increases in luminal space and tissue rearrangement would accordingly be expected in DEV tissues instead of the GRO tissues.

The marked differences in the measured mammary tissue areas between center and edge parenchyma align with the replete literature descriptions detailing heifer mammary gland growth. The greater-than-double increase in adipose tissue abundance in edge parenchyma relative to center parenchyma was expected, as edge parenchyma abuts the mammary fat pad and provides the future space for the dorsally expanding epithelial network. Center parenchyma would experience less epithelial growth and be more established compared with edge parenchyma where increased epithelial proliferation has been reported (Capuco et al., 2002; Hardy et al., 2021). Epithelial invasion into the fat pad has already occurred in center parenchyma, therefore leaving less free space for continued epithelial expansion. Accordingly, center parenchyma would contain a greater amount of lobular tissue area (collectively consisting of the individual epithelium and intralobular stromal tissue components) than edge parenchyma. Center parenchyma also contained a greater abundance of extralobular stroma than edge parenchyma, which is noteworthy and suggests extralobular stroma is providing the reinforcement for the established mammary epithelium present in center parenchyma.

The fact that the interactive effect of IMI status and parenchymal region had no effect on GRO tissue measures was unexpected and does not necessarily align with the limited literature on this topic. Nickerson and Heald (1981) examined comparable regions of parenchyma from lactating uninfected and *Staph. aureus* challenged mammary glands, and noted that more pronounced immune cell infiltration and tissue structure alterations were present in centrally located parenchyma compared with edge parenchyma in infected mammary glands. Nickerson and Heald (1981) speculated that the differences between the 2 parenchymal regions were resultant

<table>
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<th>P-value</th>
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<td>Lobular area³</td>
<td>CHALL</td>
<td>Edge</td>
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<td>0.54</td>
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</table>

1The combined tissue areas do not add to 1 mm² because different tissue areas were measured using different image sets.
2Tissue area measurements enumerated using images magnified at 100× using a 10× PlanN objective.
3Tissue area measurements enumerated using images magnified at 40× using a 4× PlanN objective.

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**Table 2.** Tissue area measures¹ for mammary tissues collected from 8 uninfected (UNINF) and 8 *Staphylococcus aureus* challenged (CHALL) mammary glands 13 d after the last estradiol and progesterone injection (i.e., DEV heifers), expressed as the area occupied (mm²) per 1 mm² of mammary tissue.
of proximity to the site of initial infection. In our study, center parenchyma was expected to be more affected by IMI because it was closer to the route of entry of infused \textit{Staph. aureus} and site of inflammation, but that was not evident. Given our mammary tissues were from nonlactating mammary glands, tissue architecture within the gland would be more homogeneous between regions, and opportunities to observe differences in tissue morphology would be less pronounced than in lactating tissues (i.e., less opportunity for luminal space to be reduced). Nevertheless, the combination of results from our study and previous studies suggest that tissue structure alterations during periods of mammary growth contribute to the reduced milk yields observed during first lactation in heifers with IMI (Hallberg et al., 1995; De Vliegher et al., 2005b).

**Evaluation of DEV Mammary Tissues**

The creation and examination of DEV mammary tissues was to delineate how IMI alters tissue structure during mammary development. Interestingly, the tissue area differences observed in GRO tissues were not present in DEV tissues. This was unexpected, as we anticipated the tissue area differences observed in GRO tissues to persist and become more pronounced with increased infection duration while the mammary tissues mature and develop. Instead, the only significant effect was that UNINF quarters had more adipose tissue area than CHALL quarters. The difference in adipose tissue areas was the most pronounced of any tissue measure evaluated in either tissue set. Trinidad et al. (1990a) similarly reported that heifer mammary quarters infected with \textit{Staph. aureus} exhibited less adipose tissue area relative to uninfected quarters; those differences were as marked in the current study. These replicated results are curious and not entirely understood. We posit that the greater abundance of adipose tissue area in UNINF quarters is a result of deeper epithelial infiltration into the mammary fat pad in UNINF quarters. This supposition suggests that CHALL mammary glands have less extensive epithelial infiltration into the fat pad. The lack of infiltration coincides with the descriptions of fibrosis (Trinidad et al., 1990a) and increased collagen abundance (Enger et al., 2020) in nonlactating mammary glands with IMI. The accumulation of excessive connective tissue could reduce the degree of epithelial branching in infected mammary glands. Impeded epithelial branching and infiltration into the fat pad in infected heifer quarters are apparent when dye is infused into the streak canal and displays limited dorsal diffusion into a chronically infected quarter relative to an uninfected quarter (Stephen C. Nickerson, University of Georgia, Athens, GA, personal communication). These factors are expected to have a negative effect on future milk production. However, further research is needed to investigate these proposed mechanisms and evaluate the roles of the mammary fat pad in the developing mammary gland during IMI.

No other quantitative morphological tissue difference areas were observed between CHALL and UN-INF quarters for DEV tissues. Several factors may be contributing to this result. First, although histologic examinations are effective in evaluating the relative cellularity and structural characteristics of the developing mammary gland, they provide no indication to the total number of mammary epithelial cells nor mammary gland mass. Assessment of overall gland growth has traditionally been assessed by measurement of total mammary DNA or udder mass. Neither is appropriate here. The leukocyte infiltration into infected mammary glands would increase total mammary DNA and mass, due to the influx of nucleated immune cells and accumulation of extracellular fluid. Further, it is not possible to definitively separate fore and rear quarters on the same udder half.

Second, we noted a marked presence of either vacuoles or lipid droplets within the epithelial cells. Even though it cannot be conclusively determined if these structures are lipid droplets or vacuoles, the presence of lipid droplets would indicate that some of the mammary glands were experiencing lactogenesis and milk secretion, whereas the presence of vacuoles would suggest that stasis of mammary secretion was stimulating some glands to begin involution. Each is plausible because alveolar-like structures were apparent in DEV tissues, coinciding with the increased luminal area, luminal contents containing secretion and fat globules, and the concurrent decrease in stromal tissue area. Indeed, estradiol and progesterone administration can initiate milk secretion with similar composition to normal milk in sexually immature heifers (Ball et al., 2000) and increase the presence of lipid droplets in hormone induced nonlactating dairy cows (Howe et al., 1975). However, if this secretion accumulates and is not removed via milking or suckling from the calf and without the continued high concentrations of estradiol and progesterone to maintain mammary structure or promote continued mammogenesis, mammary involution may be initiated and coalescing of secretory components in the epithelial cells can occur. Hallmarks of mammary involution include marked tissue structure changes that coincide with curbed secretory cell activity and vacuole formation in epithelial cells (Holst et al., 1987; Hurley, 1989). This process can be rapid, as Holst et al. (1987) noted that involution is evident as early as 2 d after cessation of milking. If these mammary glands were experiencing involution, involution

induced tissue structure changes may have masked any tissue structure changes that would result from IMI. Ultimately, whether these droplet-like structures are in fact lipid droplets or indications of vacuolation cannot be delineated, and the outlined limitations should highlight that these data be interpreted after careful consideration.

Regarding parenchymal regions, the lack of difference in tissue areas occupied by the epithelium, luminal space, and extralobular stroma between edge and center parenchyma was not surprising. A greater area occupied by adipose tissue was observed in edge parenchyma compared with center parenchymal tissue. This was expected as edge parenchyma was located near the mammary fat pad as previously discussed. Edge parenchyma also had marginally less intralobular stroma compared with center parenchyma. This may be a result of edge parenchyma containing greater epithelial proliferation than center parenchyma (Capuco et al., 2002; Hardy et al., 2021), and thereby constituting less area composed of intralobular stroma as the epithelial structures expand into the fat pad.

**General Discussions**

Variation in udder characteristics before enrollment was observed. Udders of heifers from cohort A were not producing secretions at the time of enrollment whereas some heifers screened for cohort B had some mammary secretion available for collection. Differences in lactogenic response to the hormone induction protocol between cohorts was also observed. The observed differences are suspect to be resultant of photoperiod, as photoperiod has been documented to influence the bovine mammary gland development and future milk yield of heifers (Petitclerc et al., 1985; Dahl et al., 2000; Rius and Dahl, 2006). Heifers from cohort A were attempted to be sampled during the month of April and enrolled in the study during early May, where daylight hours were lower, yet markedly increasing. Cohort A heifer mammary glands collected 13 d postinjections were distended and producing milk-like secretions, a drastic comparison to the observed mammary glands before enrollment. The heifers in cohort B began hormonal injections in June, when daylight hours were longer and more constant, and the mammary glands collected 13 d postinjections did not experience the drastic response to hormone treatment compared with the prior cohort. Consideration of the effects of photoperiod in future heifer studies may be useful to limit variability and should be reported in all future studies, given the effects photoperiod can have on mammary gland related response variables.

An important result of this study was the consistent lack of mammary secretions available for collection. We were surprised by this result because the literature is awash with descriptions of IMI prevalence in breeding age heifers (Boddie et al., 1987; Trinidad et al., 1990b; Fox et al., 1995); the collection of adequate volumes of mammary secretions would have allowed for enumeration of total and differential SCC. After several personal communications (Lawrence K. Fox, professor emeritus, Washington State University, and Stephen C. Nickerson, professor emeritus, University of Georgia), it was remarked that obtaining mammary secretion in breeding age heifers commonly used saline lavage, which is not overtly apparent in the seminal reports. Future studies should be cognizant if applying a lavage sampling technique to obtain mammary secretions samples, as this technique may increase the risk of sample contamination or increase the risk of undesirable IMI.

**CONCLUSIONS**

In our study, *Staph. aureus* challenged nonlactating mammary glands that were hormonally stimulated to grow and develop increased immune cell infiltration of both intralobular stroma and luminal compartments. This invasion was associated with changes in mammary structure during the growth phase whereby *Staph. aureus* challenged quarters exhibited reduced areas of epithelium and tended to have greater areas of intralobular stroma relative to uninfected quarters. Mammary gland development appeared partially influenced by a *Staph. aureus* IMI as uninfected quarters contained a greater tissue area occupied by adipose tissue compared with *Staph. aureus* challenged quarters. The results from this study suggest that IMI during mammogenesis limits mammary growth and alters mammary development. These tissue changes together are expected to negatively affect first lactation milk yield. Preventing and mitigating the negative consequences of these infections are important steps in improving udder health in the modern dairy industry today. Future studies seeking to evaluate the long-term effects of heifer IMI should consider following pubertal heifers into first lactation to evaluate how a prepartum IMI affects first lactation milk yield.

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