# Effect of calving season on uterine disease incidence and bacterial content of the vagina in dairy cows

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## *ABSTRACT*

Uterine disease and heat stress are recurrent problems in dairy production known to impair subsequent lactation and reproduction. To investigate how environmental temperature may impact uterine disease incidence, records of 3,507 calvings in Florida over a 5-year period were evaluated. The incidence of metritis increased from 21.1% in the cool season (October through March) to 24.2% during the warm season (April through September, $P < 0.05$). We hypothesize that seasonal variation in environmental temperature influences uterine disease incidence and severity in the dairy cow. To elucidate a link between environmental temperature and uterine disease, a total of 102 cows were enrolled during summer (September 2017; $n = 51$) and winter (February-March 2018; n = 51). Cows were maintained on pasture during the dry period and moved to free stall barns with fans and water soakers immediately prior to calving and remained in that environment after calving. Vaginal mucus was collected and graded on days 7 and 21 postpartum to evaluate the incidence of uterine disease and quantify bacterial content and species using qPCR. As expected, daily milk yield for the first 60 DIM was reduced during the summer compared with the winter (32.6 ± 1.62 vs 37.23 ± 1.60 kg, $P < 0.05$) consistent with effects of heat stress prepartum. Interestingly, a higher proportion of cows had persistent uterine disease on both d 7 and d 21 in

the summer compared with cows in the winter (58 vs 29.4 %, $P < 0.05$) and total bacterial content of the vagina was greater on d 7 compared to d 21. Vaginal content of *E. coli*, *T. pyogenes*, *F. necrophorum* and *P. melaninogenica*, bacteria known to be causative of uterine disease in dairy cows, were also quantified. Vaginal mucus on d 7 had a higher *F. necrophorum* load during the winter relative to summer, however, there were no further effects of season on total or specific pathogen load. Our data suggests that heat stress in the prepartum period related to season of calving could be impacting cow related factors that predispose the occurrence and persistence of uterine disease in the dairy cow.

***Key words:*** *heat stress, uterine disease, milk yield, tolerance, bacteria*

## *INTRODUCTION*

Postpartum uterine disease is a common problem for the dairy industry. Uterine disease is associated with decreased milk yield and reduced reproductive performance. It is estimated that 40% of cows will develop uterine disease in the postpartum period, including metritis and endometritis (Sheldon et al., 2019). Even after the resolution of clinical signs, cows with metritis produce less milk in the subsequent lactation, take longer to become pregnant and have higher abortion rates compared with healthy counterparts (Mahnani et al., 2015, Sheldon et at., 2009). Cows with endometritis have lower conception rates, increased days open and higher culling due to reproductive failure after successful treatment (LeBlanc 2008, Sheldon et al., 2009). Combining treatment cost, milk loss, decreased fertility, culling and animal replacement, uterine disease costs the United States dairy industry between $650 million and $900 million per year (Sheldon et al., 2009, Lima et al., 2019).

Uterine diseases are characterized by the presence of pathogenic bacteria in the uterus following calving that cause localized tissue damage and inflammation. Bacteria routinely cultured from cows with uterine disease include *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum,* and *Prevotella melaninogenica* (Griffin et al., 1974, Bonnett et al., 1991, Huszenicza et al., 1999, Williams et al., 2005). Nonetheless, metagenomic techniques have revealed associations between uterine disease and other bacteria phyla that are challenging to culture using standard techniques or are not conventionally associated with uterine disease, such as Bacteroidetes and Firmicutes (Machado et al., 2012, Peng et al., 2013). Epithelial and stromal cells of the endometrium detect bacteria using Toll-like receptors that result in an innate inflammatory response, characterized by increased production of proinflammatory cytokines and chemokines including interleukin (IL)-1β, IL-6 and IL-8 (Cronin et al., 2012).

In addition to the negative consequences of uterine disease on dairy production, heat stress decreases fertility and imparts negative consequences on offspring that were exposed to heat stress in utero (Negrón-Pérez et al., 2019, Ouellet et al., 2020). Heat stress of dairy cows is defined as environmental temperatures that exceed the thermoneutral zone of the cow, making it difficult for the animal to effectively dissipate heat to the environment and maintain normal body temperature. The role of environmental temperatures on the pathogenesis of uterine disease in dairy cows is unknown. There is evidence that elevated temperatures increase pathogen proliferation and predispose infectious disease incidence. For example, during summer months bacterial loads are higher in cow bedding and on teat skin compared to winter months, which is associated with an increased occurrence of mastitis (Rowbothan & Ruegg, 2016, Hogan et al., 1989). While these data suggest that elevated environmental temperatures favor increased pathogen load in the environment, it is unclear if the prevalence of uterine disease is affected by

environmental season. Increased environmental temperature could increase pathogenic bacterial prevalence or alter host immune function, both of which could impact the prevalence of uterine disease in the dairy cow. Indeed, there is evidence that prepartum heat stress impairs immune function in the post-partum period, even when heat stress abatement is available after calving (Dahl et al., 2020).

Here, we hypothesized that seasonal variation in environmental temperature influences uterine disease incidence and severity in the dairy cow. To address this hypothesis, we evaluated uterine disease incidence in lactating dairy cows at a single location in Florida during the winter and summer, while simultaneously evaluating bacterial content of the lower reproductive tract. This study determined the impact of season on uterine disease incidence, while evaluating if uterine disease was associated with seasonal variations of vaginal bacterial content in the dairy cow.

## *MATERIALS AND METHODS*

### *Influence of season on metritis incidence*

Data for retrospective analysis was obtained from the University of Florida Dairy Research Unit management software Afifarm database (SAE Afikim, Israel). The data included all calving events from January 1, 2012, through December 31, 2017 (n = 3,507). For each calving, cow ID, calving date, estimated 305-d milk production, occurrence of clinical metritis and cow's lactation number were recorded. After calving, cows were housed in a free stall barn with access to fans and soakers for heat stress abatement, whereas dry period management was on pasture. Metritis was characterized by a watery red-brown uterine discharge with foul smell and an enlarged uterus diagnosed between d 4 and d 13 after calving. Cows that aborted were

91 excluded from the analysis. Metritis incidence was calculated as the proportion of cows that
92 developed clinical metritis divided by the total cows that calved during that month.
93 Retrospective weather data was collected from the Alachua Station of the Florida
94 Automated Weather Network (FAWN, IFAS-University of Florida), located approximately 3 km
95 from the University of Florida Dairy Research Unit. Daily observation of air temperature (2 m)
96 and relative humidity from the analyzed period were used to calculate the temperature-humidity
97 index (THI) based on the equation developed by the National Research Council (1971) and
98 recommended by Dikmen and Hansen (2009):

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

99 where T = air temperature (°C) and RH = relative humidity (%). Calving between April 1 and
100 September 30 was defined as the warm period and calving between October 1 and March 31 was
101 defined as the cool period.
102
103 *Animal enrollment during winter and summer*
104 All animal procedures were approved by the University of Florida Institutional Animal
105 Care and Use Committee. To observe the development of uterine disease and assess bacterial
106 content in the lower reproductive tract, pregnant Holstein cows or heifers were sequentially
107 enrolled at the University of Florida Dairy Research Unit during the summer (September 2017; *n*
108 = 51) or winter (February to March 2018; *n* = 51). All animals were housed on pasture without
109 heat abatement until approximately 3 weeks before their expected calving date at which time
110 animals were moved to a free stall barn with fans and water soakers, receiving TMR twice a day
111 and ad libitum water. Animal health and uterine disease were assessed on d 7 and d 21
112 postpartum. Milk production data and health events until 60 DIM were collected using farm

management software (Afifarm). The average 5-day prepartum and 5-day postpartum THI were calculated for each animal using the daily reports of air temperature and relative humidity obtained from the FAWN Alachua Station.

*Collection and grading of vaginal mucus*

Vaginal mucus was sampled on d 7 and d 21 after parturition from each cow. Briefly, the vulva was thoroughly cleaned using 70% ethanol and paper towel prior to the insertion of a sterile Metricheck tool (Simcro, Hamilton, New Zealand). The Metricheck tool consists of a stainless-steel rod with a rubber collection cup on the tip. Once inserted in the vagina the tool was gently moved in a consistent manner to sample the entire vaginal canal, collecting mucus from the ventral, dorsal and lateral portions. After careful removal of the tool, the sampled content was examined and placed in a sterile bijou tube (Thermo Fisher Scientific, Waltham, MA). The mucus was graded based on the proportion of pus and scored from 0 to 4. Score 0 = clear vaginal mucus; score 1 = mucus with flecks of white pus; 2 = mucus with less than 50% white pus; score 3 = mucus with more than 50% white pus; score 4 = sanguinopurulent mucus (adapted from Sheldon et al., 2009). Cows were classified as having uterine disease (UD) if d 7 vaginal mucus grade $\geq 3$ or d 21 grade $\geq 2$. Samples were maintained on ice and transported to the laboratory and stored at -80°C for further analysis.

*Bacterial DNA isolation and quantification of bacterial content*

Total bacterial DNA was isolated from vaginal mucus samples using the DNeasy Power Soil kit (Qiagen, Germany) as previously described (Piersanti et al., 2019). Briefly, mucus samples were thawed on ice and then homogenized by vortexing for 5 min. A total of 250 mg of

each sample was added to a tube containing garnet particles and guanidine thiocyanate and placed in a tissue homogenizer (Precellys 24, Bertin Technologies SAS, France). Samples were homogenized using 3 cycles (30 s at 6,000 × *g*, 60 s pause, 30 s at 6,000 × *g*) with a 5 min incubation on ice in between each cycle. Supernatants were then collected and added to the DNeasy Power Soil spin columns for purification of DNA following the manufacturer's instructions. Purified DNA was used to quantify total bacterial content using the Femto Bacterial DNA Quantification Kit (Zymo Research, CA) that targets 16S rRNA. Briefly, real time quantitative PCR was performed using the primer mix from the commercial kit containing SYTO 9 fluorescent dye and primers targeting 16s rRNA in a 20 µL reaction containing 2 µl of total extracted DNA in each well. A CFX Connect Real-Time PCR System (Bio-Rad Laboratories) was employed using a 3-step protocol of initial denaturation at 95°C for 10 min, amplification consisting of 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. Quantification of 16S rRNA was based on the standard curve provided with the kit performed in parallel with the samples. All reactions were performed in duplicate and no-template controls were included. Results are described as nanograms of 16S rRNA per milligram of vaginal mucus. The extraction of 16S rRNA from mucus samples was validated by a spike-in/recovery method using known amounts of purified bacteria prior to the analysis of the samples. Intra-assay and inter-assay coefficient of variation were 0.3% and 2.2% respectively, with a recovery of 100.5% of expected 16S rRNA after extraction of spike-in mucus.

*Detection of specific bacteria in vaginal mucus*

158 Vaginal content of pathogenic bacteria associated with uterine disease was performed
159 using DNA isolated from mucus samples above. Specific primers for pathogens associated with
160 uterine disease, *E. coli, T. pyogenes, F. necrophorum* and *P. melaninogenica*, were designed
161 using the NCBI primer-design tool or previously published and verified by BLAST (Malinen et
162 al., 2003, Belser et al., 2015, Cunha et al., 2018) (**Table 1**). All primers were validated for
163 amplification efficiency prior to sample analysis and conformed to MIQE guidelines (Pearson
164 correlation coefficient $R^2 > 0.98$ and efficiency between 90 and 110%) (Bustin et al., 2009).
165 Quantitative real time PCR was performed in 20 µL reactions containing 18 µL of SYBR Green
166 Master Mix (Bio-Rad Laboratories, Hercules, CA) with 300 nM of each forward and reverse
167 primer and 2 µL of template DNA. PCR was performed using a CFX Connect Real-Time PCR
168 System (Bio-Rad Laboratories) with a 3-step protocol with enzyme activation at 95°C for 3 min
169 followed by amplification with 40 cycles of denaturation at 95°C for 10 s, annealing between
170 53.5 and 64.5°C for 10s depending on the primer (**Table 1**), and extension at 72°C for 30 s. A
171 melt curve was included for each reaction to ensure amplification of a single PCR product. Every
172 reaction was performed in duplicate, and a no-template control was included. Quantification of
173 specific pathogens was based on a standard curve with purified DNA from each pathogen. Total
174 DNA of *E. coli* MS499, *T. pyogenes* MS249 (supplied by Dr. Martin Sheldon, Swansea
175 University) and *F. necrophorum* (supplied by Dr. Klibs Galvão, University of Florida) was
176 extracted using the DNeasy Power Soil kit from live cultures (preparation described in Piersanti
177 et al., 2019). Purified DNA of *P. melaninogenica* was purchased from ATCC (Manassas, VA;
178 #25845). Results for each pathogen were normalized by weight of mucus and are expressed as
179 CFU/µL/mg of mucus for *E. coli* and *T. pyogenes* and pg/uL/mg of mucus for *F. necrophorum*
180 and *P. melaninogenica*.

*Statistical analysis*

All data were analyzed using SPSS Statistics v26 (IBM Corporation, Armonk, NY). Estimated 305-d milk production, and THI data pertaining to the retrospective analysis of 3,507 animals utilized a linear mixed model with pairwise comparisons between fixed effects of season, parity and the interaction between season and parity with season as a contrast field. Cow was added as a random factor. Records missing estimated 305-d milk production were excluded from this analysis. Metritis incidence was analyzed using a generalized linear mixed model for binomial distribution with fixed effects of season, parity, and the interaction of season by parity with pairwise comparisons and season as the contrast field. The effect of THI on metritis incidence was also evaluated using the same model by replacing the fixed effects of season with either the 5-day average prepartum THI or the 5-day average postpartum THI.

Continuous variables presented in **Table 2**, estimated 305-d milk production and vaginal bacterial content of the 102 animals that calved in summer or winter were analyzed using linear mixed model with fixed effects of season, parity and the interaction of season and parity with pairwise comparisons with season as the contrast field for the interaction. Cow was added as a random factor. Milk yield, ECM, and milk components were analyzed using linear mixed model with repeated measurements for daily milk production up to 60 DIM with fixed effects of season, parity, and the interaction of season by parity, and cow was included as a random effect. Categorical variables in **Table 2** were analyzed using a generalized linear mixed model for multinomial distribution with fixed effects of season, parity and the interaction of season and parity with pairwise comparisons and season as contrast field for the interaction. Data presented in **Table 3** were analyzed using linear mixed model with fixed effect of season and cow as a

random effect. The impact of metritis on daily milk yield was analyzed using a linear mixed model with fixed effects of season, parity, metritis, the interaction of season and parity with season as contrast field, and the interaction of parity and metritis with parity as contrast field. Bacterial content of the vagina was analyzed using a linear mixed model with fixed effects of season, parity, health status (cows were classified as having uterine disease if d 7 vaginal mucus grade ≥ 3 or d 21 grade ≥ 2) and the interaction of health status with season with pairwise comparisons. Statistical significance was set at $P \leq 0.05$ and tendency at $P \leq 0.08$.

## *RESULTS*

### *The incidence of metritis is increased, and milk production is decreased during warmer months in Florida*

Retrospective analysis of 3,507 calving events between 2012 and 2017 (1,364 primiparous and 2,143 multiparous) was performed to determine the effect of season (warm versus cool) on milk production and the incidence of clinical metritis. Average monthly THI was calculated using daily averages for air temperature and relative humidity from 2012 to 2018. Calving between April 1 and September 30 was defined as the warm period and calving between October 1 and March 31 was defined as the cool period. The average daily THI for the warm period (74.3 ± 0.1) was higher ($P < 0.05$) than the average daily THI for the cool period (60.8 ± 0.3). Of the 3,507 calving events analyzed, 2,204 cows had recorded 305-d milk production estimates available for analysis. Multiparous cows produced more milk than primiparous cows (9,424 ± 78 *vs* 8,613 ± 92, $P < 0.01$). Average 305-d milk production for the warm period per cow (8,819 ± 90 kg) was lower ($P < 0.01$) than the 305-d milk production during the cool period (9,219 ± 85 kg; **Fig. 1**). However, there was a significant interaction of season by parity ($P <$

227 0.01) on 305-d milk production with the effect of season restricted to multiparous cows, that
228 exhibited an 894 kg decrease in 305-d milk production in the warm period (9,871 ± 103 *vs* 8,977
229 ± 14, $P < 0.01$, **Fig. 1**). Average 305-d milk production in the warm period (8,661 ± 130 kg) and
230 cool period (8,566 ± 129 kg) for primiparous cows was not affected by season (**Fig. 1**).
231 The overall incidence of metritis from 2012 to 2017 at the University of Florida Dairy
232 Research Unit was 21.4%. The overall incidence of metritis during the warm period increased
233 (24.1%; $P = 0.035$) compared with the cool period (21.1%; **Fig. 2**). Overall, primiparous cows
234 had a higher incidence of metritis (27.7%) relative to multiparous cows (17.5%, $P < 0.01$).
235 Calving during the warm period increased the relative risk of developing metritis by 18%
236 compared with calving in the cool period (RR = 1.18, 95% CI = 1.02 to 1.31). The incidence of
237 metritis in primiparous cows was 29.5% during the warm period and 26% during the cool period
238 ($P = 0.15$). The incidence of metritis in multiparous cows was 18.7% in the warm period and
239 16.2% in the cool period ($P = 0.13$). There was no effect of the 5-day average prepartum THI or
240 5-day average postpartum THI on metritis incidence, regardless of parity.
241

242 *The effect of calving season on milk production, cow performance and uterine disease incidence*

243 A total of 51 cows calving in the summer (September, 2017; 5-day postpartum average
244 THI = 78.6 ± 0.5) and 51 cows calving in the winter (February to March, 2018; 5-day postpartum
245 average THI = 64.4 ± 0.6) were enrolled to monitor daily milk production, evaluate cow
246 performance, observe the development of uterine disease, and assess bacterial content of the
247 lower reproductive tract (**Table 2**). As expected, the 5-day average prepartum and postpartum
248 THI were increased ($P < 0.01$) during the summer relative to winter. Overall, milk production
249 and energy corrected milk (ECM) were increased in multiparous cows relative to primiparous

cows ($P < 0.01$) and milk production tended to be reduced during summer compared with winter ($P = 0.06$) whereas ECM was similar between seasons (**Table 3**). For multiparous cows, milk production was decreased in cows calving in the summer relative to winter, while for primiparous cows the production was the same between seasons. Overall, the percent milk protein was not affected by parity, and it was increased ($P < 0.01$) in cows calving in the summer compared with those calving in the winter; conversely, absolute milk protein yield was increased ($P < 0.01$) in multiparous versus primiparous cows but did not differ between seasons. Overall, percent milk fat was increased ($P < 0.01$) in cows that calved during the winter compared with summer. Absolute milk fat yield was increased ($P = 0.01$) in cows that calved in the winter due to the increase in fat yield in milk produced by multiparous cows ($P = 0.02$). Multiparous cows had a higher ($P < 0.01$) absolute lactose yield compared with primiparous cows but there was no effect of calving season. Percent milk lactose was higher for multiparous cows ($P < 0.01$), and it was increased in cows calving in the summer ($P < 0.01$).

Overall, gestation length of cows calving in the winter and summer was the same, as was the gestation length of primiparous and multiparous cows. Intriguingly, primiparous cows had a longer gestation ($P < 0.01$) during the summer (275.92 ± 3.82 days) compared with winter (269.15 ± 5.05 days). The incidence of twin pregnancy, retained placenta, ketosis, and other diseases in the first 60 DIM was not affected by calving season. There was a tendency ($P = 0.06$) for an increased culling of cows that calved during the winter compared to cows that calved during the summer, because of an increase in culling primiparous cows that calved in the winter ($P = 0.05$). Overall, the interval from calving to first insemination was increased ($P < 0.01$) by > 8 days in cows that calved in the summer, while the number of inseminations to achieve a pregnancy was not affected by season.

The overall incidence of clinical metritis during the summer (19.6%; 10/51) and winter (25.5%; 13/51) was the same (**Table 1**, $P > 0.05$). When considering the effects of clinical metritis on milk production, daily milk yield was affected ($P < 0.05$) by parity, clinical metritis, season and by the interaction between parity and clinical metritis (**Fig. 4**). There was no effect of the interaction between season and clinical metritis on daily milk production for the first 60 DIM. Cows with no clinical metritis that calved in the winter produced the greatest milk yield whereas and cows with clinical metritis that calved in the summer produced the lowest milk yield.

Based on vaginal mucus grade, cows were categorized as having uterine disease (UD) if d 7 vaginal mucus was ≥ grade 3, or if d 21 mucus was ≥ grade 2, resulting in four groups of animals: 1) cows that were free of uterine disease on d 7 and d 21; 2) cows with uterine disease on d 7 which was resolved by d 21, 3) cows that were free of uterine disease on day 7 and developed uterine disease by d 21; and 4) cows that had uterine disease on both d 7 and d 21 (**Fig. 4**). The proportion of cows with no uterine disease on both d 7 and d 21 did not differ between cows calving in the winter (27.5%) and the summer (19.6%); however, the proportion of cows with uterine disease on both d 7 and d 21 was greater ($P < 0.05$) in cows that calved in the summer (58.0%) compared with those that calved in the winter (29.4%), suggesting recovery from uterine disease on d 7 was reduced in cows that calved in the summer (**Fig. 4**).

*The effect of calving season on bacterial content in the lower reproductive tract*

Total bacterial 16S rRNA in vaginal mucus at d 7 postpartum was greater in abundance relative to d 21 (5.64 ± 1.33 *vs* 1.26 ± 0.50 ng/mg mucus, **Fig. 5A**, $P < 0.05$). There was no effect of calving season on total bacterial 16S rRNA at d 7 or d 21 ($P = 0.65$ and $P = 0.11$,

296 respectively). Total bacterial 16S rRNA was affected by vaginal mucus grade collected on d 7 or
297 d 21 ($P < 0.05$), with total bacterial 16S rRNA increasing with vaginal mucus grade **(Fig. 5B-C**,
298 $P < 0.05$). On d 21 there was an effect of calving season on total bacterial 16S rRNA ($P < 0.05$);
299 however, this was primarily driven by a single cow with grade 4 mucus that calved in the winter
300 with an exceptionally high 16S rRNA content.

301 Known pathogens associated with uterine disease were quantified in vaginal mucus using
302 real time RT-PCR with primers designed for *E. coli*, *T. pyogenes*, *F. necrophorum* and *P.*
303 *melaninogenica* (**Fig. 6 and 7**). The content of *E. coli*, *T. pyogenes*, *F. necrophorum* or *P.*
304 *melaninogenica* in vaginal mucus was the same at d 7 and d 21 regardless of calving season (**Fig.**
305 **6**). However, the content of *T. pyogenes* in vaginal mucus at d 7 was reduced ($P = 0.04$) in cows
306 that calved in the summer compared to those that calved in the winter and tended ($P = 0.07$) to be
307 reduced at d 21 in cows calving in the summer compared with the winter (**Fig. 6B**).

308 Using the same classification for uterine disease described above (d 7 vaginal mucus
309 grade $\geq 3$ or d 21 grade $\geq 2$), cows with uterine disease at d 7 (**Fig. 7A-E**) or d 21 (**Fig. 7F-J**) had
310 increased ($P < 0.01$) total bacterial 16S rRNA content in vaginal mucus compared to cows with
311 no uterine disease, but there was no effect of calving season. Vaginal mucus content of *E. coli* at
312 d 21 was increased ($P < 0.05$) in cows with uterine disease compared to cows with no uterine
313 disease (**Fig. 7B**), but not at d 7 (**Fig. 7G**). Calving during the winter increased ($P < 0.05$)
314 vaginal mucus content of *T. pyogenes* in cows with uterine disease compared to cows with no
315 uterine disease at d 7 (**Fig. 7C**) and 21 (**Fig. 7H**). Vaginal mucus content of *F. necrophorum* at d
316 7 was increased ($P < 0.05$) in cows with uterine disease compared to cows with no uterine
317 disease (**Fig. 7D**), but not at d 21 (**Fig. 7I**). Surprisingly, vaginal mucus content of *P.*

*melaninogenica* at d 7 tended to decrease ($P = 0.07$) in cows with uterine disease relative to cows with no uterine disease (**Fig. 7E**), but this tendency was not observed at d 21 (**Fig. 7J**).

## *DISCUSSION*

Although the negative impacts of heat stress and uterine disease on milk yield and reproduction have been described in the dairy cow, there is a lack of evidence for a link between elevated environmental temperatures and reproductive tract health. Here, we reviewed records of over 3,000 animals that calved during a 5-year period at the University of Florida and found an increased incidence of metritis during warmer months and recapitulated the findings of seasonal variation in milk production. In addition, we investigated if the increased incidence of uterine disease seen during the warmer months was due to an increase in bacterial content of the lower reproductive tract by evaluating vaginal mucus samples from postpartum cows during the winter and summer. While our data confirmed that total bacterial 16S RNA, *E. coli*, *T. pyogenes* and *F. necrophorum* content is increased in cows with uterine disease, the season of calving does not influence bacterial content of vaginal mucus. However, cows that calved in the summer did not recover from uterine disease as effectively as cows that calved in the winter. This suggests that environmental factors associated with season, such as heat stress, might affect host factors that can predispose cows to the development and poorer recovery of uterine disease, including altering the immune response to pathogens or decreasing a cows ability to tolerate pathogens.

In agreement with our findings, Gernand et al., (2019) reported an increased incidence of uterine disorders during summer associated with increased THI by using a robust model to correct for additional environmental factors. Conflicting results have been presented regarding the influence of seasonality in the incidence of uterine diseases where seasonality had no impact

in the incidence of uterine diseases (Thompson and Dahl, 2012, Pinedo et al., 2020) or the higher incidence of disease occurred during the winter (Markusfeld, 1984, Benzaquen et al., 2007). These discrepancies in association might be explained by differences in management, housing conditions, diet composition and to the arbitrary definition of season between studies. For our retrospective analysis, we split the year evenly between 6 months of warm season and 6 months of a cold season in the sub-tropical region of north central Florida. This definition meant each month within the warm season had an average THI > 68 which is documented to facilitate the outward signs of heat stress in high producing dairy cows (Zimbelman et al., 2009, De Rensis et al., 2015).

The THI is a widely utilized indicator of heat stress that combines environmental temperature and relative humidity, since elevated humidity reduces heat dissipation to the environment (Dikmen & Hansen, 2009). The herd utilized here was located in Florida, a subtropical region with the second highest relative humidity in the US. The decreased milk production observed in the warm season is consistent with these cows being under heat stress conditions in late gestation and lactation (Kadzere et al., 2002, Fabris et al., 2019). In our observational study using a smaller number of cows we also observed a tendency for reduced milk production in the summer and an increase in days open, characteristic of cows under heat stress. A limitation of the current study was our inability to isolate the effects of heat stress from other seasonal factors. While data presented here were collected from cows under the same management strategy at the same location, other factors affecting the observed outcomes may include changes in photoperiod, water and air quality, feed composition and dry matter intake.

Establishment of disease requires an interaction between a susceptible host, a pathogen and the environment that influences both host and pathogen. Seasonal changes in the

environment can change host or pathogen factors and predispose individuals to disease by various mechanisms, including increased pathogen proliferation, changes in host behavior, increased transmission within a population and change in host susceptibility (Altizer et al., 2006). Cow under heat stress conditions alter immune system function, likely reducing immune capacity which leaves animals vulnerable to infection (Dahl et al., 2020). Epidemiological studies show that some infectious diseases have defined seasonal outbreaks explained in part by changes in pathogen and/or host factors that are influenced by environmental temperature (Dowell, 2001, Altizer et al., 2006). Rates of bloodstream infections in humans caused by Gram-negative bacteria are associated with higher environmental temperatures and have a higher prevalence during summer compared to winter. For example, *Actinobacter spp.* infections are increased by 51.8% and *E. coli* infections are increased by 12.2% in patients during the summer compared with the winter (Eber et al., 2011). However, our findings show that total bacterial load in the lower reproductive tract is not influenced by season. The lack of seasonal effect on bacterial content of vaginal mucus in conjunction with increased uterine disease incidence suggests that host factors rather than dysbiosis may cause changes in disease incidence and persistence.

Immune resilience mechanisms that act to limit the establishment of disease include avoidance, resistance, and tolerance (Sheldon et al., 2020). Avoidance mechanisms limit host exposure to pathogens, while resistance mechanisms actively reduce pathogen load, and tolerance mechanism limit tissue and cellular damage caused by pathogens. The data described here suggests that the vaginal content of total bacteria and other recognized pathogens it not affected by calving season; nonetheless, calving during the summer increased the incidence and persistence of uterine disease. This suggests that heat stress in the summer could impair cow

resistance or tolerance to pathogens, increasing tissue damage and predisposing cows to the establishment of uterine disease. Studies show that heat stress impacts the immune system of dairy cows depending on the physiological state of the cow when exposed to heat stress. Dry period heat stress decreased neutrophilic function during lactation, while PBMCs from lactating dairy cows exposed to heat stress have increased production of TNFα and IL-10 in response to LPS (do Amaral et al. 2011, Marins et al., 2021). Sheldon et al. (2019) described an observation involving increased metabolic stress and a decrease in tolerance due to high production where cows producing > 35 kg milk/d were described as having decreased uterine health relative to cows that produced < 35 kg milk/d; however, the pathogen content of the uterus was comparable between cows of high and low production value cows, suggesting host tolerance was compromised in the high producing animal. Tolerance mechanisms which may contribute to reducing uterine disease include physical barriers to infections such as the epithelium or mucus, neutralization of pathogen toxins, tissue repair mechanisms or adaptive metabolic responses (Sheldon et al., 2020). For example, during calving a large proportion of the protective endometrial epithelium is lost, effectively exposing the upper reproductive tract to the environment and potential endometrial tissue damage. Underlying endometrial stromal cells are more sensitive to the cholesterol-dependent cytolysin of *T. pyogenes* compared with endometrial epithelial cells, which would permit greater endometrial tissue damage during uterine infection if the epithelium is lost during parturition (Amos et al., 2014). Further investigation of the effect of seasonality on immune function was not performed in this study and are needed to further determine if seasonality or heat stress is impacting immune resilience mechanisms.

The process of immune resistance is often intertwined with immune tolerance and together they mediate pathogen control. Uterine disease triggers a classical innate immune

response characterized by increased secretion of pro-inflammatory cytokines including IL-1α, IL-1β and IL-6 in the uterus (Kim et al., 2014), effectively increasing host resistance. In parallel, cellular response to heat shock is characterized by increased synthesis of heat shock proteins (HSP), a family of chaperones activated in response to cellular damage (Sorensen et al., 2003, Schiaffonati & Tiberio, 1997). Mammalian HSP can form complexes with antigenic peptides and activate cells of the innate immune system and increase secretion of pro-inflammatory cytokines (Srivastava et al., 1998, Kol et al., 1999, Moroi et al., 2000, Basu et al., 2000, Basu & Srivastava, 2000). Interestingly, heat-stressed bovine endometrial epithelial cells reduce production of IL-6 compared with epithelial cells cultured in thermoneutral conditions, conversely endometrial stromal cells increase IL-6 production relative to stromal cells cultured in thermoneutral conditions (Sakai et al., 2020). Therefore, given the immunomodulatory capacity of heat stress, combined with unaltered pathogen content of the vagina observed here, a cytokine disbalance may be an important factor in altering the response of heat-stressed cows to pathogens, especially from late gestation to early lactation. Moreover, persistent inflammation caused by prolonged heat stress may generate tissue damage and interfere with tolerance mechanisms that could be involved in maintaining uterine health or promoting disease resolution. Further investigation is necessary to disentangle the mechanisms by which heat stress impacts uterine defense mechanisms in the bovine.

## *CONCLUSIONS*

Our data demonstrate an increased incidence and poorer recovery of uterine disease during the warm season. In parallel, there was no association between pathogen content of the lower reproductive tract and season of calving. Combined, this new knowledge suggests that host

resilience may be affected by season and alter the development, progression, and recovery from uterine disease in the high producing dairy cow.

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583 ***FIGURE LEGENDS***

584 **Figure 1.** Effect of calving season and parity on 305-d milk yield in cows from 2012 to 2017.
585 Estimated 305-d milk production was calculated for a total of 2,204 cows (multiparous, *n* =
586 1,354 and primiparous, *n* = 850) during the warm (April through September) and cool (October
587 through March) seasons. Bars represent the mean ± S.E.M. * $P \leq 0.05$ for comparisons within
588 each group.

589

590 **Figure 2. Effect of calving season on metritis incidence.** The average monthly incidence of
591 clinical metritis was calculated from 2012 to 2017 (*n* = 3,507) by dividing the number of cows
592 that developed metritis by total number of cows that calved in each month between January 1
593 2012 and December 31 2017 at the University of Florida Dairy Research Unit. The solid black
594 lines represent the monthly average incidence of clinical metritis for primiparous (●) and
595 multiparous (●) cows ± S.E.M. The dashed line (○) represents the average monthly temperature
596 humidity index (THI) ± S.E.M.

597

598 **Figure 3. Effect of calving season and clinical metritis on daily milk yield.** Average daily
599 milk yield for the first 60 DIM from cows that calved during the winter (*n* = 51) or the summer
600 (*n* = 51). Circles represent cows that did not develop clinical metritis after calving in the winter
601 (●) or summer (●). Triangles represent cows that did develop clinical metritis after calving in the
602 winter (▲) or summer (▲). Data are presented as mean kg per day ± S.E.M.

603

604 **Figure 4. The effect of season on uterine disease incidence at day 7 and day 21 postpartum.**
605 Vaginal mucus was collected on day 7 and day 21 postpartum from cows that calved during the

606 winter ($n$ = 51) or the summer ($n$ = 51) and graded from 0 to 4 according to pus content. Cows
607 were categorized as having uterine disease (UD) on d 7 if vaginal mucus was ≥ grade 3, or if d
608 21 mucus was ≥ grade 2, and then assigned to 4 different groups: no UD d 7 and d 21; UD on d 7
609 and no UD on d 21; no UD on d 7 and UD on d 21, UD on d 7 and on d 21. Bars represent the
610 percentage of cows in each category according to season. * $P \leq 0.05$ for comparisons within each
611 group.
612
613 **Figure 5. Total bacterial content of vaginal mucus according to the day of collection and**
614 **vaginal mucus grade.** Total bacterial content of vaginal mucus collected from cows that calved
615 during the winter ($n$ = 51) or the summer ($n$ = 51) was quantified by targeting bacterial 16S
616 rRNA using the Femto Bacterial Quantification Kit. Data are presented comparing d 7 and d 21
617 collections (A) or according to mucus grade collected on d 7 (B) or d 21 (C). Quantification was
618 based on the provided standard curve and expressed as ng of targeted DNA per mg of mucus.
619 Bars represent the mean ± S.E.M. * $P \leq 0.05$ compared to indicated group.
620
621 **Figure 6. Quantification of specific bacteria in vaginal mucus according to the day of**
622 **collection and season of calving.** (A) *E. coli*, (B) *T. pyogenes*, (C) *P. melaninogenica* and (D) *F.*
623 *necrophorum* were quantified using real time RT-PCR in vaginal mucus samples collected on d 7
624 and d 21 postpartum from cows that calved during the winter ($n$ = 51) or the summer ($n$ = 51).
625 Quantification was based on a standard curve for each bacterium and is expressed as CFU/uL per
626 mg of mucus for *E. coli* and *T. Pyogenes* and as pg/uL per mg of mucus for *P. Melaninogenica*
627 and *F. Necrophorum*. Dots represent individual cows. * $P \leq 0.05$ compared to indicated group.
628

**Figure 7. Bacterial content of vaginal mucus according to uterine health status.** (A, F) Total 16S rRNA content, (B, G) *E. coli*, (C, H) *T. pyogenes*, (D, I) *F. necrophorum* and (E, J) *P. melaninogenica* were quantified using real time RT-PCR in vaginal mucus samples collected on d 7 (A-E) and d 21 (F-J) postpartum from cows that calved during the winter ($n$ = 51) or the summer ($n$ = 51). Cows were categorized as having uterine disease (UD) based on vaginal mucus grade (uterine disease if d 7 vaginal mucus was ≥ grade 3, or if d 21 mucus was ≥ grade 2). Data are expressed as ng per mg of mucus for total 16S rRNA, CFU/uL per mg of mucus for *E. coli* and *T. pyogenes*, and pg/uL per mg of mucus for *P. melaninogenica* and *F. necrophorum*. * $P \leq 0.05$ for comparison between health status, superscript $^{a,b}$ indicate $P \leq 0.05$ between health status within season, superscript $^{y,x}$ indicates $0.05 \leq P \leq 0.08$ between health status within season.

Table 1. PCR primers used for real time RT-PCR.

| Bacteria (target gene) | Primer sequence (5'-3') | Annealing Temperature (ºC) | Design |
|---|---|---|---|
| *E. coli* (*16S*) | F–GTTAATACCTTTGCTCATTGA<br>R–ACCAGGGTATCTAATCCTGTT | 53.5 | Malinen et al., 2003 |
| *T. Pyogenes* (*plo*) | F–GGCCCGAATGTCACCGC<br>R–AACTCCGCCTCTAGCGC | 64.5 | Belser et al., 2015 |
| *F. Necrophorum* (*ikta*) | F–GATTGGGGGATAGCGGTAAT<br>R–GAGCCTCCACATTTAGTCGC | 63.0 | Cunha et al., 2018 |
| *P. Melaninogenica* (*phyA*) | F–ACAAAGAGGCAAACCAAGCG<br>R–TACGAAGCATCCGTTCAGGG | 55.0 | In-house design |

Table 2. Descriptive table of cows enrolled during winter and summer.

| | All Cows[1] | | *P*-value | | |
|---|---|---|---|---|---|
| | Winter | Summer | Season | Parity | Season × Parity |
| Cows | 51 | 51 | - | - | - |
| Primiparous | 15 | 27 | - | - | - |
| Multiparous | 36 | 24 | - | - | - |
| Lactation number | 2.37 ± 1.31 | 1.86 ± 1.15 | 0.75 | - | - |
| Gestation length[2] | 273.68 ± 6.86 | 274.50 ± 5.74 | 0.11 | 0.11 | < 0.01 |
| Twins | 8% (4/51) | 4% (2/51) | 0.73 | 0.10 | 1 |
| RP | 6% (3/51) | 0 (0/51) | 0.10 | 1 | 0.10 |
| Avg daily milk (kg) | 41.36 ± 14.45 | 35.35 ± 11.24 | 0.06 | < 0.01 | 0.38 |
| Avg daily ECM | 40.27 ± 9.80 | 34.46 ± 7.41 | 0.11 | < 0.01 | 0.58 |
| Milk protein (kg) | 0.99 ± 0.34 | 0.96 ± 0.31 | 0.66 | < 0.01 | 0.81 |
| Milk protein (%) | 2.57 ± 0.36 | 2.79 ± 0.35 | < 0.01 | 0.28 | 0.31 |
| Milk fat (kg) | 1.45 ± 0.51 | 1.22 ± 0.41 | 0.01 | < 0.01 | 0.61 |
| Milk fat (%) | 3.82 ± 0.78 | 3.57 ± 0.68 | 0.01 | 0.45 | 0.46 |
| Milk lactose (kg) | 1.53 ± 0.65 | 1.46 ± 0.53 | 0.98 | < 0.01 | 0.42 |
| Milk lactose (%) | 3.90 ± 0.76 | 4.13 ± 0.56 | < 0.01 | < 0.01 | 0.09 |
| Metritis incidence[3] | 25% (13/51) | 20% (10/51) | 0.17 | 0.24 | 0.21 |
| Ketosis incidence[3] | 67% (34/51) | 51% (26/51) | 0.13 | 0.89 | 0.56 |
| Other disease[3,4] incidence | 35% (18/51) | 25% (13/51) | 0.12 | 0.05 | 0.45 |
| Culled[3] | 12% (6/51) | 2% (1/51) | 0.06 | 0.85 | 0.54 |
| Days to first insemination | 77.40 ± 2.87 | 69.38 ± 11.96 | < 0.01 | 0.86 | 0.98 |
| Insemination: pregnancy[5] | 2.88 ± 2.02 | 2.68 ± 1.62 | 0.62 | 0.46 | 0.20 |
| Vaginal mucus grade (d 7) | 2.65 ± 1.35 | 3.10 ± 1.19 | < 0.01 | 0.94 | 0.93 |
| Total 16S content (d 7) | 4.66 ± 11.51 | 6.52 ± 13.74 | < 0.01 | 0.14 | 0.08 |
| Vaginal mucus grade (d 21) | 1.55 ± 1.08 | 1.92 ± 1.21 | 0.13 | 0.05 | 0.14 |
| Total 16S content (d 21) | 1.18 ± 3.01 | 1.39 ± 6.15 | 0.53 | 0.69 | 0.88 |
| Lactation number | 2.37 ± 1.31 | 1.86 ± 1.15 | 0.33 | 0.04 | 0.51 |
| Gestation length | 273.68 ± 6.86 | 274.50 ± 5.74 | 0.71 | 0.20 | 0.44 |

[1]All variables are shown as mean ± S.E.M.

[1]Variables were tested for the effect of season, parity and the interaction between parity and season.

[2]For gestation length cows that had an abortion or stillbirth were excluded.
[3]Metritis, ketosis, other diseases and culling were analyzed up to 60 DIM.
[4]Other diseases included mastitis, respiratory problems, foot problems and displaced abomasum.
[5]Insemination : Pregnancy was analyzed until cow was diagnosed pregnant or up to 200 DIM.

Table 3. Descriptive table from cows enrolled during winter and summer sorted by parity.

| | Primiparous Cows[1] | | | Multiparous Cows[1] | | |
|---|---|---|---|---|---|---|
| | Winter | Summer | Season | Winter | Summer | Season |
| Cows | 15 | 27 | - | 36 | 24 | - |
| Lactation | - | - | - | 2.94 ± 1.14 | 2.83 ± 1.00 | 0.62 |
| Gestation Length[2] | 269.15 ± 5.05 | 275.92 ± 3.82 | < 0.01 | 275.41 ± 6.72 | 273.08± 6.97 | 0.33 |
| Twins | 0 | 0 | - | 11% (4/36) | 8% (2/24) | 0.54 |
| RP | 0 | 0 | - | 8% (3/36) | 0 | 0.21 |
| Avg daily milk (kg) | 33.59 ± 10.98 | 31.54 ± 9.06 | 0.56 | 44.56 ± 14.49 | 39.73 ± 11.90 | 0.02 |
| Avg daily ECM | 33.43 ± 7.08 | 30.91 ± 3.92 | 0.50 | 43.08 ± 9.37 | 38.54 ± 8.31 | 0.09 |
| Milk protein (kg) | 0.86 ± 0.28 | 0.87 ± 0.25 | 0.66 | 1.07 ± 0.35 | 1.07 ± 0.33 | 0.87 |
| Milk protein (%) | 2.63 ± 0.38 | 2.79 ± 0.35 | < 0.01 | 2.54 ± 0.34 | 2.78 ± 0.36 | < 0.01 |
| Milk fat (kg) | 1.25 ± 0.41 | 1.10 ± 0.30 | 0.22 | 1.57 ± 0.53 | 1.36 ± 0.47 | 0.02 |
| Milk fat (%) | 3.87 ± 0.68 | 3.60 ± 0.66 | 0.04 | 3.79 ± 0.83 | 3.53 ± 0.71 | 0.17 |
| Milk lactose (kg) | 1.23 ± 0.50 | 1.29 ± 0.43 | 0.61 | 1.71 ± 0.67 | 1.66 ± 0.57 | 0.51 |
| Milk lactose (%) | 3.75 ± 0.84 | 4.07 ± 0.60 | < 0.01 | 3.99 ± 0.70 | 4.21 ± 0.47 | < 0.01 |
| Metritis incidence[3] | 20% (3/15) | 26% (7/27) | 0.49 | 28% (10/36) | 13% (3/24) | 0.14 |
| Ketosis incidence[3] | 60% (9/15) | 52% (14/27) | 0.43 | 70% (25/36) | 50% (12/24) | 0.11 |
| Other disease incidence[3,4] | 47% (7/15) | 37% (10/27) | 0.39 | 31% (11/36) | 13% (3/34) | 0.09 |
| Culled[3] | 13% (2/15) | 0 | 0.05 | 11% (4/36) | 4% (1/24) | 0.35 |
| Days to first insemination | 77.69 ± 2.14 | 69.59 ± 8.82 | 0.01 | 77.28 ± 3.14 | 69.22 ± 15.04 | < 0.01 |
| Insemination: pregnancy[5] | 2.73 ± 2.19 | 3.04 ± 1.53 | 0.61 | 2.94 ± 1.97 | 2.26 ± 1.66 | 0.17 |
| Prepartum THI | 2.73 ± 1.10 | 3.52 ± 0.80 | < 0.01 | 2.61 ± 1.46 | 2.63 ± 1.38 | < 0.01 |
| Postpartum THI | 5.14 ± 15.35 | 7.21 ± 11.65 | < 0.01 | 4.46 ± 9.74 | 5.74 ± 16.00 | < 0.01 |
| Vaginal mucus grade (d 7) | 2.00 ± 1.00 | 2.07 ± 1.27 | 0.05 | 1.36 ± 1.07 | 1.75 ± 1.15 | 0.97 |
| Total 16S content (d 7) | 0.82 ± 2.26 | 0.41 ± 1.40 | 0.62 | 1.33 ± 3.29 | 2.49 ± 8.81 | 0.62 |
| Vaginal mucus grade (d 21) | 15 | 27 | 0.84 | 36 | 24 | 0.20 |
| Total 16S content (d 21) | - | - | 0.79 | 2.94 ± 1.14 | 2.83 ± 1.00 | 0.36 |

[1]All variables are shown as mean ± S.E.M.

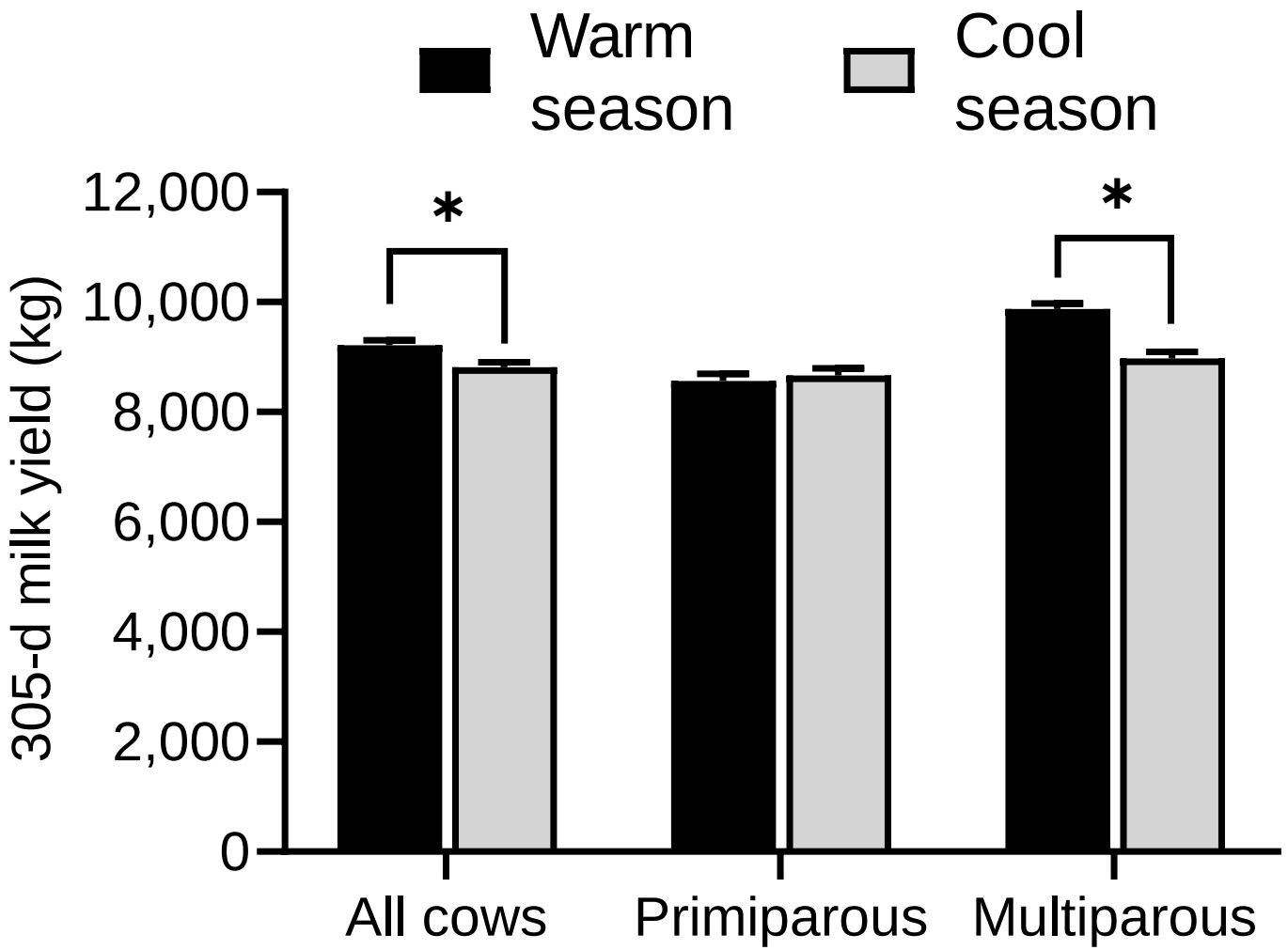
[1]Variables were tested for the effect of season and the interaction between parity and season.

[2]For gestation length cows that had an abortion or stillbirth were excluded.

[3]Metritis, ketosis, other diseases and culling were analyzed up to 60 DIM.

[4]Other diseases included mastitis, respiratory problems, foot problems and displaced abomasum.
[5]Insemination : Pregnancy was analyzed until cow was diagnosed pregnant or up to 200 DIM.

**Figure 1**




Season < 0.01
Parity < 0.01
Season*Parity < 0.01

**Figure 2**

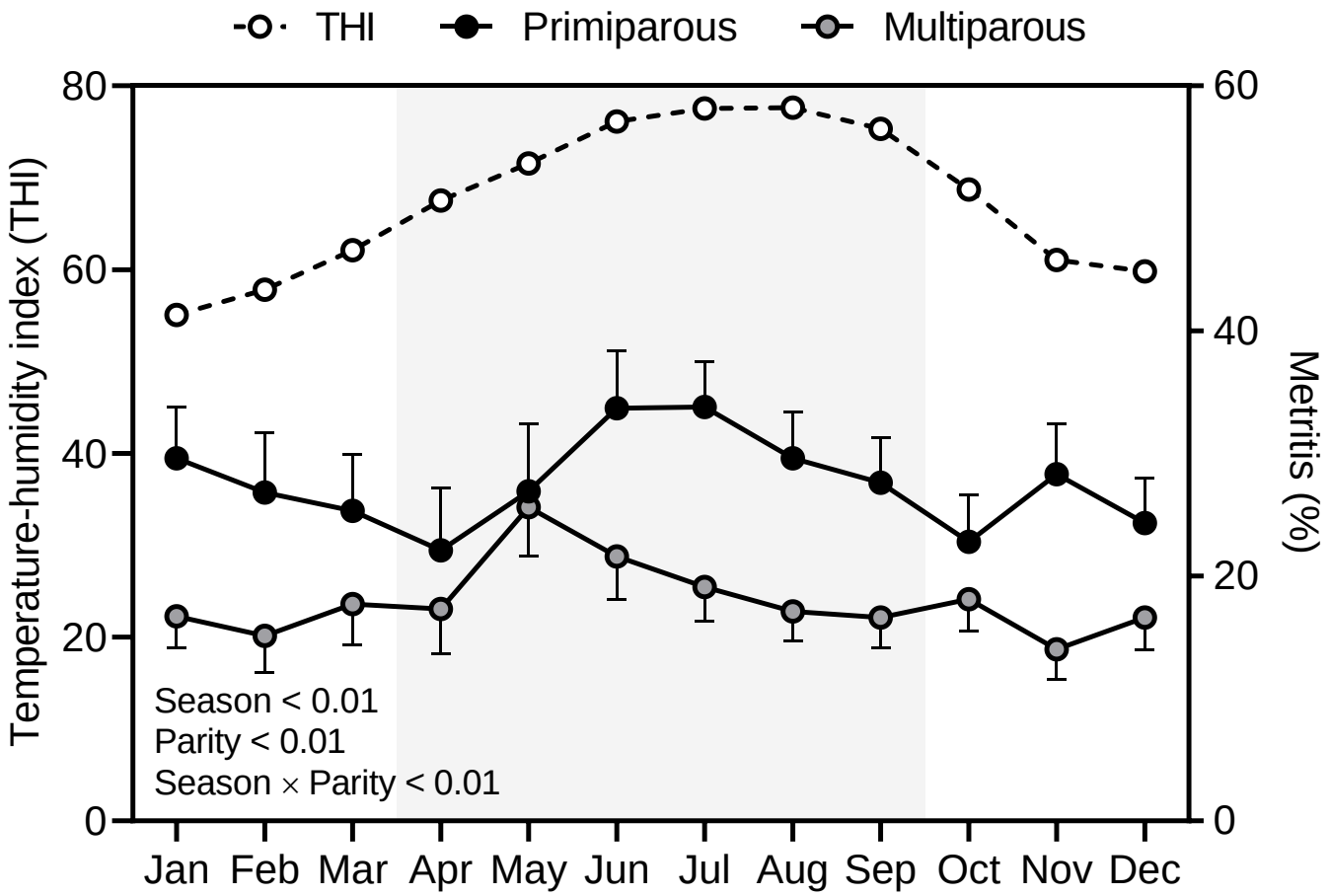

THI
Primiparous
Multiparous
Temperature-humidity index (THI)
Metritis (%)
80
60
40
20
0
60
40
20
0
Season < 0.01
Parity < 0.01
Season × Parity < 0.01
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec

**Figure 3**

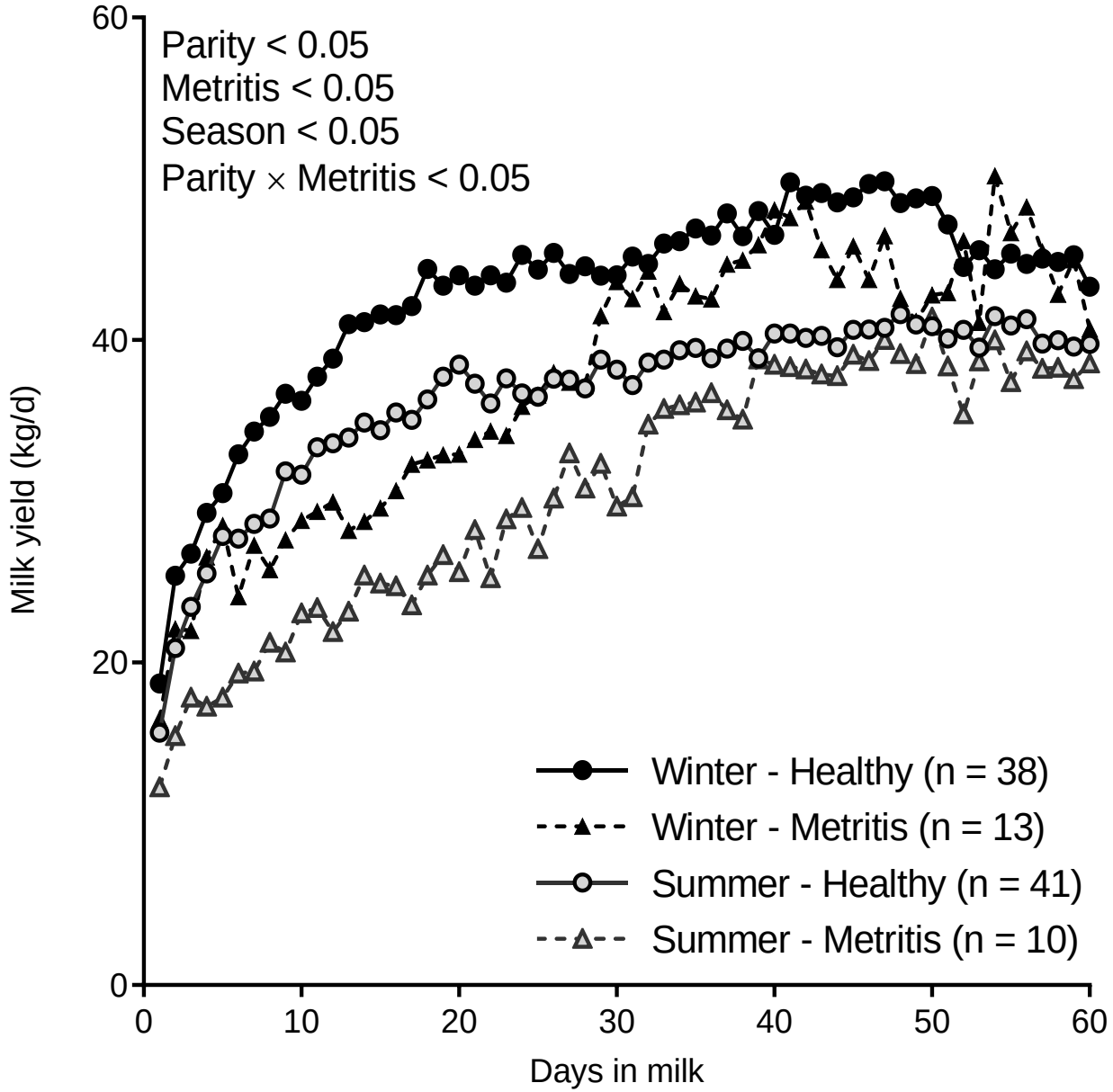

Parity < 0.05
Metritis < 0.05
Season < 0.05
Parity × Metritis < 0.05
Milk yield (kg/d)
Days in milk
0
20
40
60
0
10
20
30
40
50
60
Winter - Healthy (n = 38)
Winter - Metritis (n = 13)
Summer - Healthy (n = 41)
Summer - Metritis (n = 10)

**Figure 4**

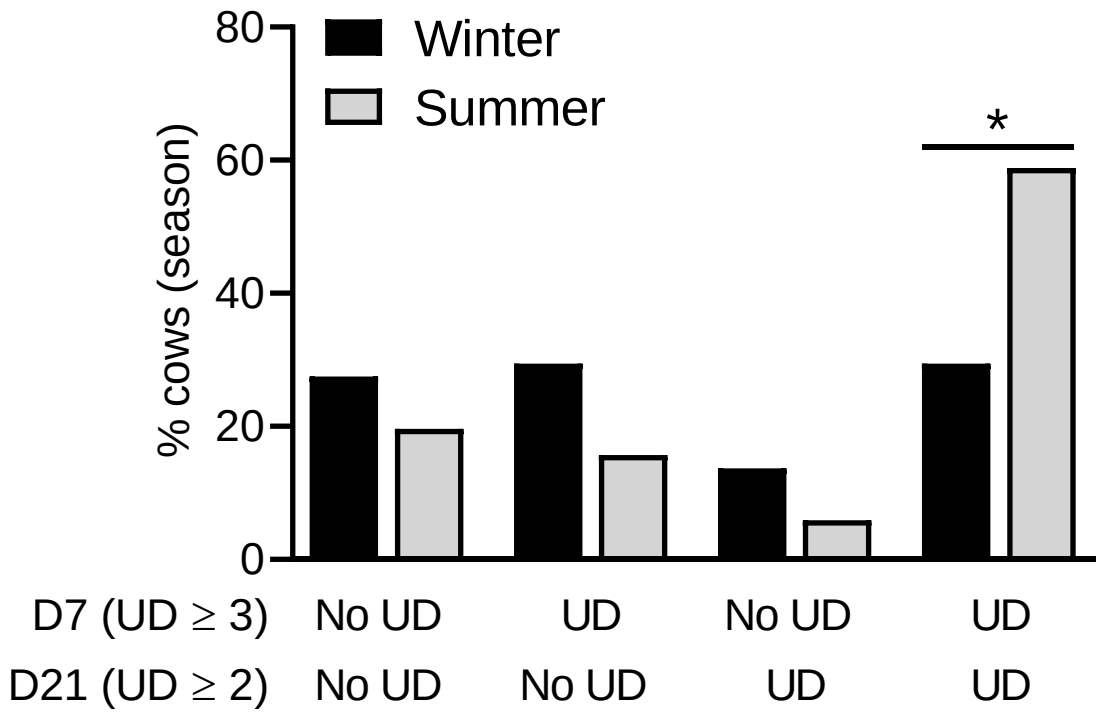

80
60
40
20
0
% cows (season)
Winter
Summer
*
D7 (UD ≥ 3)
No UD
UD
No UD
UD
D21 (UD ≥ 2)
No UD
No UD
UD
UD

**Figure 5**

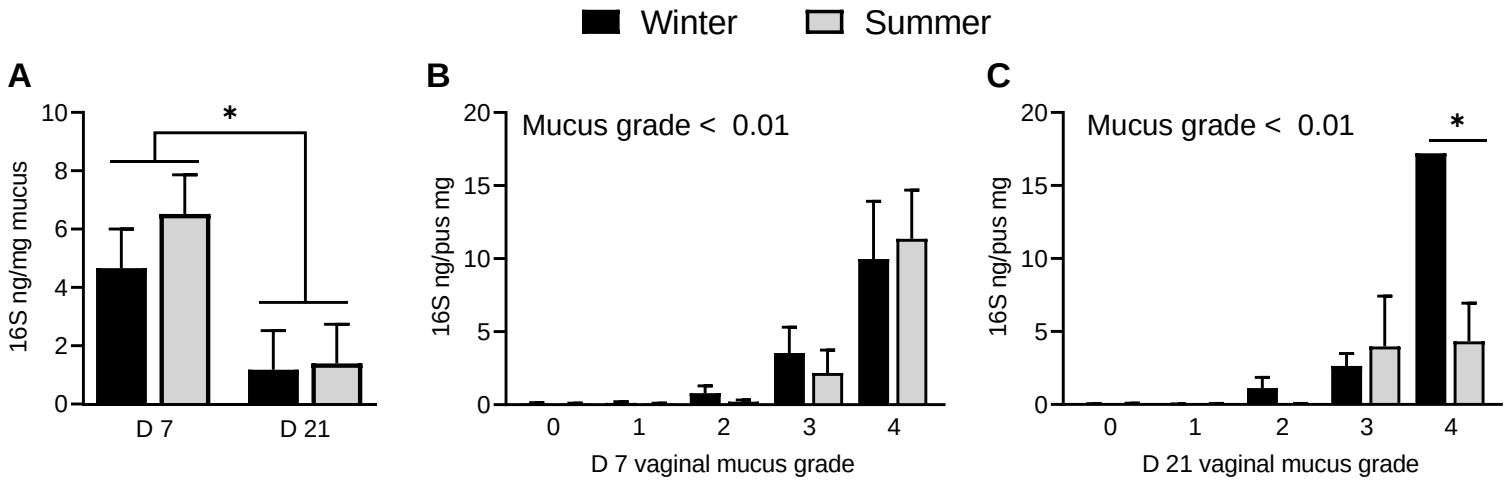

Winter
Summer
A
B
C
16S ng/mg mucus
16S ng/pus mg
Mucus grade < 0.01
Mucus grade < 0.01
*
*
D 7
D 21
D 7 vaginal mucus grade
D 21 vaginal mucus grade

**Figure 6**

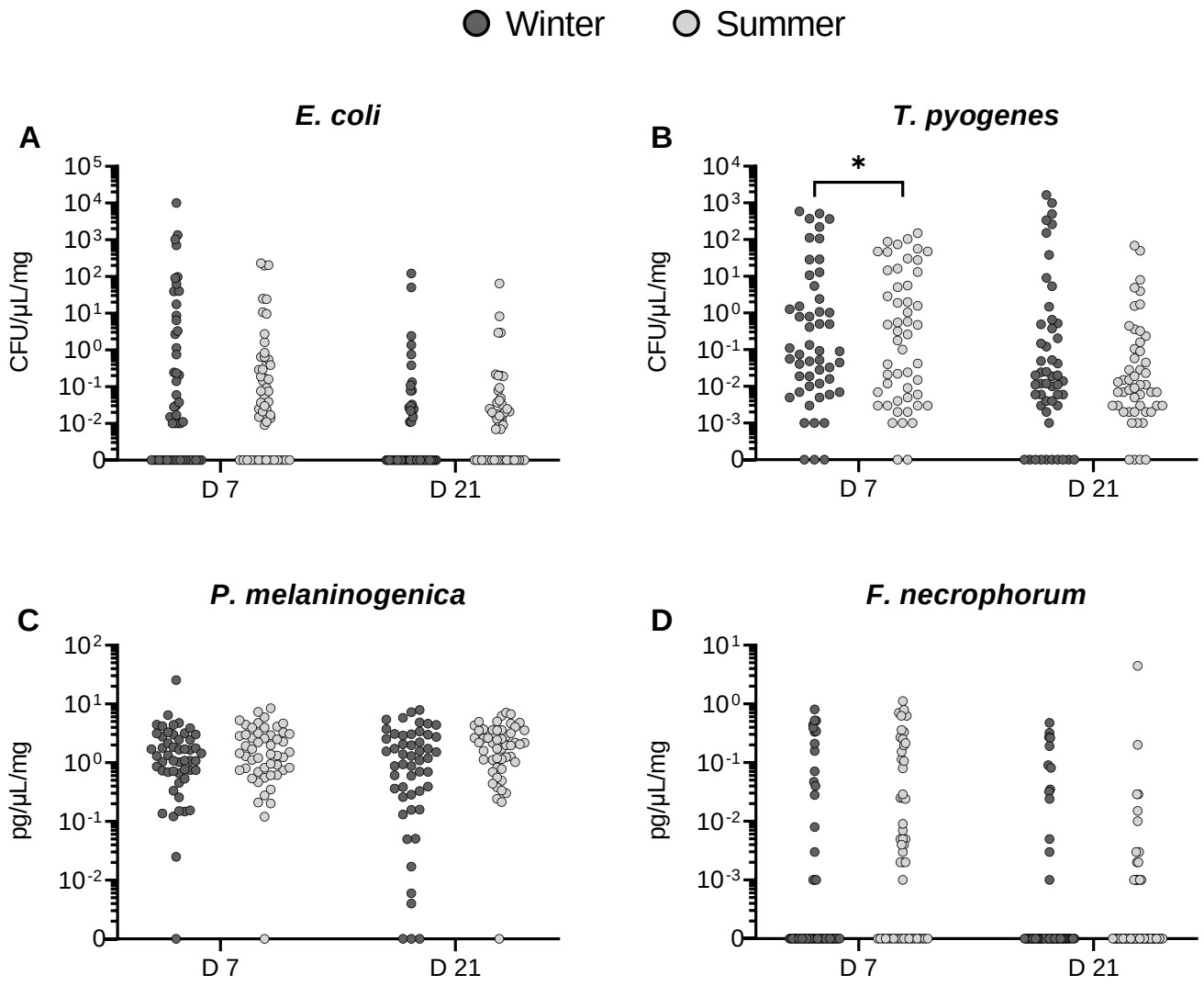
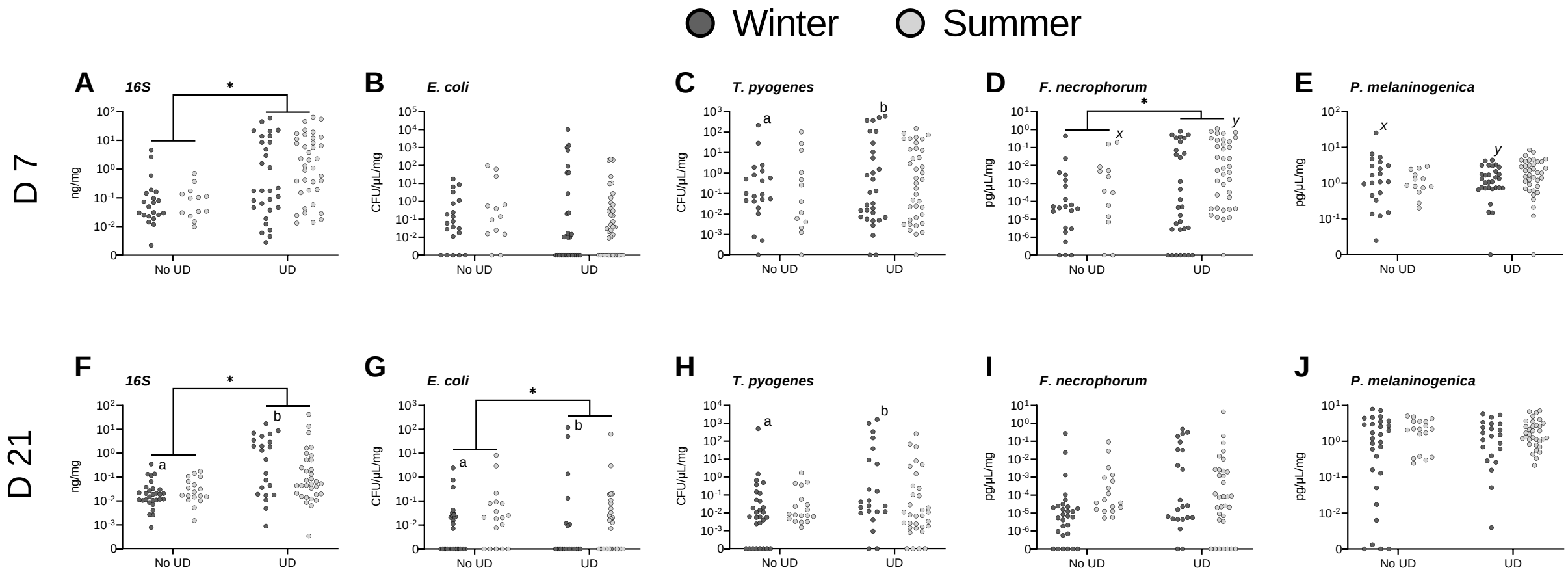

Winter
Summer
A
E. coli
CFU/µL/mg
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
$10^{-1}$
$10^{-2}$
0
D 7
D 21
B
T. pyogenes
*
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
$10^{-1}$
$10^{-2}$
$10^{-3}$
0
D 7
D 21
C
P. melaninogenica
pg/µL/mg
$10^2$
$10^1$
$10^0$
$10^{-1}$
$10^{-2}$
0
D 7
D 21
D
F. necrophorum
pg/µL/mg
$10^1$
$10^0$
$10^{-1}$
$10^{-2}$
$10^{-3}$
0
D 7
D 21

**Figure 7**


Winter
Summer
D 7
D 21
A
16S
ng/mg
B
E. coli
CFU/µL/mg
C
T. pyogenes
CFU/µL/mg
D
F. necrophorum
pg/µL/mg
E
P. melaninogenica
pg/µL/mg
F
16S
ng/mg
G
E. coli
CFU/µL/mg
H
T. pyogenes
CFU/µL/mg
I
F. necrophorum
pg/µL/mg
J
P. melaninogenica
pg/µL/mg
No UD
UD
*
a
b
x
y