DISSERTATION

# EVALUATION OF NOVEL STRATEGIES FOR IMPROVING PREVENTION AND EARLY DIAGNOSIS OF HEALTH DISORDERS IN ORGANIC DAIRY CATTLE

Submitted by

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ABSTRACT

EVALUATION OF NOVEL STRATEGIES FOR IMPROVING PREVENTION AND EARLY DIAGNOSIS OF HEALTH DISORDERS IN ORGANIC DAIRY CATTLE

The research projects covered in this dissertation were carried out under organic certified dairy management systems and were intended to provide basic information on the implementation of strategies for nutritional management, prevention and early detection of disease on dairy cows. Organic dairy systems provide unique settings to perform research on animal health and productivity, since certified organic dairy farms are regulated by fixed requirements that must be met at all times. Conversely to conventional dairy farms, the standardization of the regulations by the national organic program causes that all organic certified dairy farms have similar challenges regarding animal health, reproductive and productive performance. Therefore, significant evidence generated in studies carried out on organic dairy animals could be transversally applied into certified organic and conventional dairy farms in the USA, as production restrictions are increasing for both systems. Another relevant reason for implementing research studies for improving animal health and productivity for organic dairy systems is the growth of organic food sales in the USA, where organic dairy products represent the 15% of the organic food market. Due to this growth, the number of organic dairy cows per herd has consistently increased during the last 10 years, which will require more research to understand the relationship between dairy animals' performance and intensive organic systems.

This dissertation presents five research studies performed in young heifers and in lactating dairy cows, with emphasis 0n supplementation of energy dense feeds, cow's conditioning behaviors, and in the development of methods of data analysis for improving welfare assessment and disease detection, based on behavioral multivariable correlations. The study presented in Chapter I evaluates the effects of supplemental rumen-protected fats (**RPF**), specifically developed for certified organic dairy farms in the USA. A randomized controlled trial was performed using 202 Holstein cows supplemented once a day with 0.45 kg/head of RPF (n = 101) or a control diet (n = 101) from calving day until 150 days in milk (**DIM**). The evaluated outcomes included daily milk yield, milk components, reproductive performance, metabolic markers, culling and mortality. A significant effect after the inclusion of RPF was found in daily milk yield among multiparous (**MP**) cows, where supplemented cows had greater milk yield (1.5 kg/d) compared to MP control cows by 150 DIM. No effect was found in primiparous (**PP**) cows. Reproduction performance was not improved or impaired by RPF in the diets. The inclusion of RPF tended to increase serum concentrations of β-Hydroxybutirate and non-esterified fatty acids especially at 7 and 21 DIM, which agreed with published studies on RPF supplementation in conventional dairy farms. Cows supplemented with RPF tended to have lower culling risk but mortality risk did not differ among supplemented and control cows.

The research presented in Chapter II has direct relationship with the previous study, as manual sorting of the two treatment groups was used to separate the study cows for the RPF supplementation. A case report of the self-sorting behavior acquired by the study cows and the investigation of the conditioning factor for such behavior is presented. To test the effectiveness of human sorting on separating subgroups of lactating dairy cows and to assess the level of conditioning to this activity, we compared three sorting methods applied to the same cows: 1)

human active sorting (AS) at the pen gate; 2) human presence as passive sorting (PS); and 3) non-human gate sorting (GS). We hypothesized that after a training period cows become conditioned to human sorting. Holstein cows (N = 176; parity = 2.5 ± 1.3), housed within the same lactating group were randomly assigned into two subgroups (A = 91 animals and B = 85 animals) to be sequentially separated by three sorting methods (AS; PS; and GS). Each sorting method was applied once per day after morning milking during 5 days. When AS was applied, the total proportion of animals correctly sorted was of 99.8%, whereas PS had 94.8% of sorting accuracy ($P < 0.0001$). Non-human GS could not be accurately assessed because the cows lost their self-sorting behavior overcrowding one side of the pen making impossible the data collection. During the RPF study and during the evaluation of sorting methods, we observed a clear self-sorting behavior in response to human sorting, regardless the use of AS or PS. Therefore, after a period of training, lactating dairy cows became operant conditioned to human sorting, which represents an opportunity for animal separation without intense human labor or practices that result in increased animal stress.

The studies presented in chapters III and IV develop exploratory methodologies for analyzing behavioral data recorded by remote sensor devices (**RSD**) in individual dairy cows. Such data include the assessment of active time, rumination, and eating time, and locomotion and lying behavior. In chapter III, temporal relationships between two behavioral variables were evaluated considering culling status in a subset of cows classified as cases (n =12, culled cows) and healthy controls (n=30). The analysis suggested to investigate temporal relationship based on time series and cross-correlation analysis. The data presented in chapter III suggest that there are differential patterns in animals that will be culled due to health reasons and animals that remained healthy and in good productive and reproductive standing by mid-lactation.

These differential patterns in cross-correlations were observed before and after calving and predicting behavior variables for fluctuations observed in other behavior variables were determined by culling status. Additionally, in Chapter III, a relationship between production levels in the previous and current lactation and the lying time was determined. In general terms, high producing cows spent more time lying down compared to low producing cows during the first 21 d of lactation. Moreover, increments in lying times during that period were associated with increments in milk yield during the current lactation.

Chapter IV covers the development of welfare indicators based on behavioral parameters continuously evaluated by RSD. To evaluate the association between welfare status and differences in behavior, we developed a welfare status criterion based on the absence of clinical disease during the observation period (calving to 150 DIM), cyclicity, and productive performance. Animals having absence of clinical disease, cycling before 60 DIM and in or above the average from group's milk yield had significantly higher rumination and eating time, especially during the first 21 DIM compared with animals without the aforementioned conditions. Active time was not associated with the proposed welfare status. Additionally, it was determined that 5 min increments in rumination and eating time were associated with increased odds of being classified as animals in good welfare standing by 150 DIM. These findings open new research perspectives to develop welfare indicators for real-time welfare assessments without human error and in normal productive settings.

Finally, in Chapter V, we evaluated the use of sunlight reflection technology to reduce temperature in the polyethylene hutched used to raise pre-weaned dairy calves. This evaluation was performed to provide scientific evidence on the usefulness of aluminized covers already in the market; therefore, farmers can have a more informed investment decision in this type of products.

The objective was to evaluate the effect of aluminized reflective hutch covers on health and performance of pre-weaned Holstein heifers during summer. Health, behavior, rectal temperature, and respiratory rate were assessed twice per week from 1 to 60 d of life on calves housed in covered or uncovered control polyethylene hutches. No differences between treatments were found in presentation of clinical dehydration, nasal and eye discharge, rectal temperature, respiratory rate, and weight gain. However, calves in covered hutches had greater occurrence of diarrhea and abnormal ear scores. The use of reflective covers was only able to reduce the temperature of the hutch wall in intimate contact with the cover. Nonetheless, no differences were found in the center of the hutch or in the sand used for the bedding.

As the body of knowledge on organic certified dairy systems advances, new opportunities for researching the effects of novel management strategies on animal health and, productive and reproductive performance appear. Additionally, the development of new therapeutic and preventative strategies for infectious, metabolic, and obstetric diseases, as well as the implementation of innovative management especially created for organic dairy cattle, bring a new prospective in veterinary medicine and livestock science research, that will follow the line of this dissertation.

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# CHAPTER 1 - EFFECT OF SUPPLEMENTAL ORGANIC RUMEN-PROTECTED FAT ON PRODUCTIVITY, REPRODUCTIVE PERFORMANCE, METABOLIC STATUS AND CULLING IN ORGANIC DAIRY COWS

## Introduction

In organic dairy farming, as in conventional production systems, the transition period has major relevance regarding nutritional managements that may lead to a successful or unsatisfactory lactation. There are different definitions among authors about what stage on lactation should be denominated transition. Although most authors agree that transition occurs during the periparturient period, Grummer (1995), Herdt (2000) and Carvalho (2014) refer to the 3 weeks before to the 3 weeks after parturition, as the most accurate definition. The weeks prior calving are characterized for rapid fetal growth, colostrogenesis, and mammary development, besides increased activity in pathways favoring mobilization of fat and other nutrients. On the other hand, during postpartum dairy cows are challenged by nutrient deficits to support milk production, which triggers nutrient reserves mobilization of fat, labile protein, and calcium (Lean et al., 2013). These metabolic changes, combined with suboptimal dry matter intake (**DMI**), increase the risk of concomitant health disorders that occur disproportionately in a short period of time (Drackley, 1999; Duske et al., 2009). Health disorders with higher incidence reported in the US during transition include milk fever (5 to 7%; Goff, 2008), subclinical ketosis (22.4 to 55.7%; McArt et al., 2011), retained fetal membranes (4% after a normal calving; Hooshmandabbsi et al., 2018), metritis (18.5 to 27.6%; Santos et al., 2015) and displaced abomasum (3.5%; Caixeta et al., 2018). These disorders have adverse effects on milk production, reproduction, animal welfare and farm profitability.

Nutritional management in transition cows is commonly reported as a preventative strategy (Goff, 2008; Monteiro et al., 2017; Katthi et al., 2017). In this sense, dietary approaches to maintain health status of transitioning cows, and therefore preventing health disorders that affect productive and reproductive performance, should have a holistic view of the cow's metabolism supporting the energy, protein and calcium metabolism, besides the immune and rumen function (Lean et al., 2013). Nonetheless, most research has studied negative energy balance (**NEB**) and negative nutrient balance (**NNB**) separately through controlled trials of supplementation of individual feed additives that target each of those negative balances.

Energy balance can be defined as the difference between net energy intake and net energy expenditure for maintenance and milk production (Van Knegsel et al., 2005). Despite the differences in the feed additives or different diets investigated, the responses measured from the lactating cows are quite consistent across the published studies. Most studies aim to test the effects of dietary strategies on overcoming the NEB and the low DMI during the first 4 weeks after calving, providing carbohydrates, amino acids, and fats as the main fuel nutrients (Herdt, 2000; Lean et al., 2013). Ruminants, especially dairy animals, have higher requirements of interstitial glucose to synthetize large amounts of lactose for milk production. In the rumen, dietary carbohydrates are quickly metabolized to synthetize volatile fatty acids (**VFA**) that sustain the lactation, maintenance and growth, therefore, ruminants have limited carbohydrate absorption from the gut (Herdt, 2000). Glucose availability is a precondition for high milk production in dairy cows (Lohrenz et al., 2010). Increased glucose requirements to support lactation, suboptimal DMI, and NEB state cause mobilization of lean protein tissues to synthesize carbohydrates, since glucose cannot be directly metabolized from fat (Herdt, 2000).

This catabolic status, turns lipolysis as the main energy source (Lean et al., 2013; Duske et al., 2009) through β-oxidation of non-esterified fatty acids (**NEFA**), providing Acetyl-CoA as key intermediate for the Kreb cycle and as a precursor of ketone bodies (acetate, acetoacetate and β-hydroxybutyrate [**BHB**]), which are important sources of fuel for many tissues, including brain, heart and skeletal muscle (Garret and Grisham, 2007). However, the exacerbated lipid metabolism releases inflammatory mediators, causes excess of oxidative free radicals, elevated ketone bodies and decreases ruminal and blood pH, which are associated to increased risk of transition diseases (Khatti et al., 2017; Lean et al., 2003).

As indicated above, the adaptation to the NEB after parturition plays a key role in the success of the initiating lactation. In consequence, providing readily usable sources of energy to fresh cows (cows within 21 days after calving) is the main strategy adopted by farmers and it is of great interest to researchers. Due to the limited energy content of carbohydrates, a large amount of such feeds would be needed to satisfy the ruminal fermentation that leads to VFA synthesis and lactogenesis. Moreover, as previously stated, the feed intake is insufficient to meet requirements for milk production and maintenance (Duske et al., 2009). Therefore, increasing energy density per gram of feed becomes an important opportunity to overcome NEB in transition dairy cows.

Fats are energetically denser than carbohydrates. The energy density of fats is considerably higher than that of glucose. While 1 g of fat contains 8.84 Kcal, glucose contains 3.82 Kcal/g (Garret and Grisham, 2007). For example, the complete oxidation of one palmitic acid yields 106 molecules of ATP, while one molecule of glucose through glycolysis yields 2 ATP molecules and pyruvate for the TCA cycle (Garret and Grisham, 2007). The readily usable energy has determined that the use of supplemental fats and oils become a standard practice (Drackley, 1999). Nonetheless, extensive research has shown that even at low levels of supplementation, fats

decrease the DMI, depress ruminal fiber digestion and are likely to produce fatty acid isomers that cause milk fat depression (Palmquist and Jenkins, 2017). In general terms, addition of supplemental fat at levels of < 3% of the dry matter has been considered as standard when supplementing vegetable fatty acids and does not affect milk fat content (Onetti et al., 2001; Stoffel et al., 2015). However, to overcome NEB usually more energy is needed and dairy producers are sometimes tempted to increase fat content in the diets. Unfortunately, dietary fat in large amounts does not suppress lipid mobilization during transition (Drackley, 1999) that may lead to digestive problems and impaired rumen function (Hammon et al., 2008).

In the decade of 1980, a pioneer study by Palmist and Conrad (1980), motivated by the interest of enhancing the dietary energy from the use of fat, first introduced the concept of rumen inert-fat or rumen-protected fats (**RPF**). In this study, the authors noted that calcium affected the digestibility of dietary fiber. In addition, preformed calcium salts (soaps) of fat improved the fat digestibility because fats can resist biohydrogenation in the rumen (Mattos et al., 2000; Jenkins and McGuire, 2006) and be absorbed in the small gut. Since then, the use of RPF became a widely used strategy to increase energy density of the rations without negative effect on rumen function (Palmquist and Jenkins, 1980; Lohrenz et al., 2010; Hammon et al., 2008; Blum et al., 1999). Early research after the introduction of RPF showed that the addition of fatty acids as calcium salts increased forage cell wall digestibility in the rumen and gut (Palmquist and Jenkins, 2017).

Although most authors agree in that the supplementation of RFP increases milk yield and milk lactose (Pappritz et al., 2011; Lohrenz et al., 2010; Hammon et al., 2008; McNamara et al., 2003), there is controversy about the effect of RPF on DMI, protein and fat in milk, glucose turnover, hepatic function, metabolites dynamics, and reproduction performance. Opposite conclusions have been reached during the last 10 years, and this debate unveils that more research

is needed to provide thorough information for producers about the effectiveness of RPF in transition cows.

Rumen protected fats represent an alternative to increase dietary energy density for transition dairy cows (Hammon et al., 2008). Currently, most RPF contain calcium soaps of C16:0 and C18:1 fatty acids (Lohrenz et al., 2010). Megalac® (Volac Wilmar Feed Ingredients Ltd. Hetfordshire, UK), containing calcium salts of palm fatty acids and calcium salts of methionine, and Megapro Gold®, containing calcium salts of palm fatty acids and extracted rapeseed meal and whey permeate, are RPF commercially available for conventional dairy cows in the US. The suggested dose range between 0.4 and 1.5 kg/d per cow. However, few studies have performed controlled trials using commercial RPF. NcNamara et al. (2008), established that supplementation of transition cows with Megalac and Megapro Gold increased milk yield over the first 12 weeks of lactation. Another study performed by Tyagi et al (2010), determined that Megalac supplemented at 2.5% of DMI increased milk production over the first 90 days in milk (**DIM**). Additionally, the information provided by the RPF manufacturers advocates enhancement of productive and reproductive performance in transition dairy cows.

This information has gained the attention of organic dairy farmers interested in using RPF as strategy to improve energy balance, performance, and health of organic dairy cows. Nonetheless, organic dairy farming does not allow the use of the above-mentioned products since they are not included in the list of approved products for organic producers (OEFFA, 2016). Recently, Organilac (**ORG**) a RPF containing palm oil and whey protein, was approved for use in organic dairies (USDA Organic), with a dose range of 0.25 to 0.45 kg/d. However, there is no controlled research performed in commercial organic dairy farm to test the effect of ORG on the performance and health of organic lactating dairy cows.

*Study hypothesis and general objective*

We hypothesized that the supplementation of ORG from calving until 150 DIM would increase milk yield, milk solids, reproduction performance, energy metabolites and health status of organically managed Holstein cows. The objective of this study was to test the efficacy of ORG (0.45 kg/d) to improve milk yield and milk components, reproduction performance, energy metabolites profile and reduce culling and mortality in organic Holstein cows supplemented from 1 to 150 DIM.

**Materials and methods**

Study design, animals and management

The Institutional Animal Care and Use Committee at Colorado State University reviewed and approved all procedures that the study animals underwent for this trial (Protocol ID: 16-6704AA). A randomized blocked controlled trial to evaluate the effect of an organic RPF on the performance and health of transition dairy cows was conducted from January to July 2016 in an organic certified dairy farm, located in Northern Colorado. Two-hundred and two pregnant non-lactating Holstein cows were randomly selected to conform two study groups; one supplemented with organilac (ORG) and one control group (CON). The sampling frame considered a list of 800 cows in the pre-partum (close-up) group within 21 to 15 d to the expected calving day. Upon random selection, the study cows were blocked by parity (primiparous [**PP**] and multiparous [**MP**] ≥ 2 lactations), and randomly assigned into two study groups. 1) ORG group, supplemented with 1.5 Kg of a treatment pellet formulated to contain 0.45 Kg of the organic RPF (Organilac, Organic Animal Nutrition, Boulder, CO. Pellets were elaborated by Ranch-Way Feeds, Fort Collins, CO). 2) CON group, supplemented with 1.05 Kg of a control pellet formulated to match all feed components except ORG (Table 1). Both study groups had 30% of PP cows. The enrollment was

carried out in the maternity group, where cows were linked to their previously assigned treatment group.

Two color links, red for ORG group and green for CON group, attached to the identification ear-tag defined the group separation upon arrival to the fresh pen. Daily feeding of the treatment pellets delivery began within the same day of calving at the experimental pen (Figure 1), after the morning milking (07:00 h) and continued until 150 DIM. The corresponding amount of pellet was offered individually in front of the cows on top of the TMR, while they were restrained in the headlocks. The formulation and delivery of the TMR was consistent for both ORG and CON throughout the trial.

After calving, both study groups shared the same facilities, milking times (07:00, 15:00 and 23:00 h) and management at all times. However, for delivery of feeding treatment the research pen was divided into two sub pens separating ORG from CON cows, only after the morning milking. Farm personnel at the pen entrance gate performed animal separation as depicted in Figure 1. The sorting procedure was assisted by the color links and by collars only attached to control cows. After sorting, cows remained locked up consuming the treatment diets. Additionally, all the study and management related procedures were performed at that time. After 1 h of restraining, the feed bunk was cleaned, the cows were released and the temporary gates were opened (Figure 1), allowing the cows walk freely in the research pen, sharing all the other farm activities.

**Table 1.** Ingredient and nutrient composition of the treatment (ORG) and control pellets fed to the study groups during the nutritional trial. Both groups consumed the same total mixed ration (**TMR**)

| Composition | Supplement pellet | | Organilac 200 | TMR |
|---|---|---|---|---|
| | Treatment | Control | | |
| Ingredients, % of DM | | | | |
| Dehydrated ground alfalfa | 20 | 20 | - | - |
| Rumen protected fat | 30 | - | 93.6 | - |
| Ground corn | 40 | 70 | - | - |
| Molasses | 5 | 5 | - | - |
| Nonfat powder milk | 5 | 5 | - | - |
| Corn silage | - | - | - | 16 |
| Hay | - | - | - | 41.2 |
| Ray ranch grass | - | - | - | 3.3 |
| Farm grain mix | - | - | - | 33 |
| Cottonseed | - | - | - | 6.2 |
| Chemical composition, % of DM | | | | |
| DM, % | 90.3 | 85.6 | 93.6 | 14.8 |
| CP | 9.3 | 13.4 | 0.7 | 14.8 |
| Soluble Protein, % of CP | 14.1 | 13.4 | 0.2 | 5.5 |
| ADF protein, % of CP | 7.5 | 0.5 | 0.34 | 1.22 |
| NDF protein, % of CP | 10.2 | 1.72 | 0.54 | 2.51 |
| ADF | 7.9 | 10 | 1.2 | 25.3 |
| NDF | 12.8 | 20 | 1.9 | 34.8 |
| Lignin | 2.98 | 2.41 | 0.44 | 5.6 |
| Starch | - | - | - | 22.6 |
| Crude Fat | 28.6 | 5.1 | 87.5 | 4.59 |
| NE Lactation, Mcal/lb of DM | 1.26 | 0.84 | 2.36 | 0.72 |
| NE Maintenance, Mcal/lf of DM | 1.3 | 0.88 | 2.49 | 0.73 |
| NE Gain, Mcal/lb of DM | 0.94 | 0.58 | 1.86 | 0.45 |
| Ca | 3.84 | 0.87 | 9.63 | 0.85 |
| P | 0.26 | 0.33 | 0 | 0.3 |
| Mg | 0.17 | 0.2 | 0.09 | 0.3 |
| K | 0.69 | 0.94 | 0.69 | 1.47 |
| Na | 0.09 | 0.08 | 0.03 | - |
| Fe, PPM | 204 | 124 | 269 | - |
| Mn, PPM | 32 | 23 | 21 | - |
| Zn, PPM | 29 | 25 | 6 | - |
| Cu, PPM | 9 | 6 | 3 | - |

*Blood sampling and measurement of blood metabolites*

Blood samples were collected from the coccygeal vein within 24 h after calving and at 3, 7, and 21 DIM for determination of glucose, BHB and NEFA. Due to cost constrains at least 50% of the animals per group were randomly selected for laboratory analysis. Venipuncture was performed using the vacutainer system in tubes without anticoagulant (BD Vacutainer, Franklin Lakes, NJ). After collection, blood was allowed to clot for 1 h at 4$^{o}$C and then centrifuged at 2800 rpm for 15 min. Supernatant was recovered and stored at -20$^{o}$C until lab analysis. Glucose (mg/dL) and BHB (mmol/L) was determined using an electronic handheld meter (FreeStyle Optimum, Abbot Diabetes Care Ltd, Witney, UK) as referenced by Voyvoda and Erdogan et al (2010), showing a sensitivity and specificity of 85 and 94%.

Non-esterified fatty acid (mEq/L) concentration was determined using a colorimetric enzymatic assay (NEFA-HR [2], Wako Chemicals, Richmond, VA). This assay consisted in the preparations of the provided color reagents A and B and the five standards (NEFA concentrations 0,125, 500, and 1000 uEq/L). In 96-well flat bottom plate, 4 uL of the negative control, standards and sample were pipetted in duplicates. Next, 225 uL of the color reagent A were added to each well and incubated at 37$^{o}$C for 20 minutes. After incubation, 75 uL of the color reagent B were added to each well and incubated another 20 minutes at 37 $^{o}$C. Finally, the absorbance of the plate was read in a microplate reader at 550 nm and the NEFA concentration was calculated from the standards using linear regression (Synergy HT, Biotek, Winooski, VT).

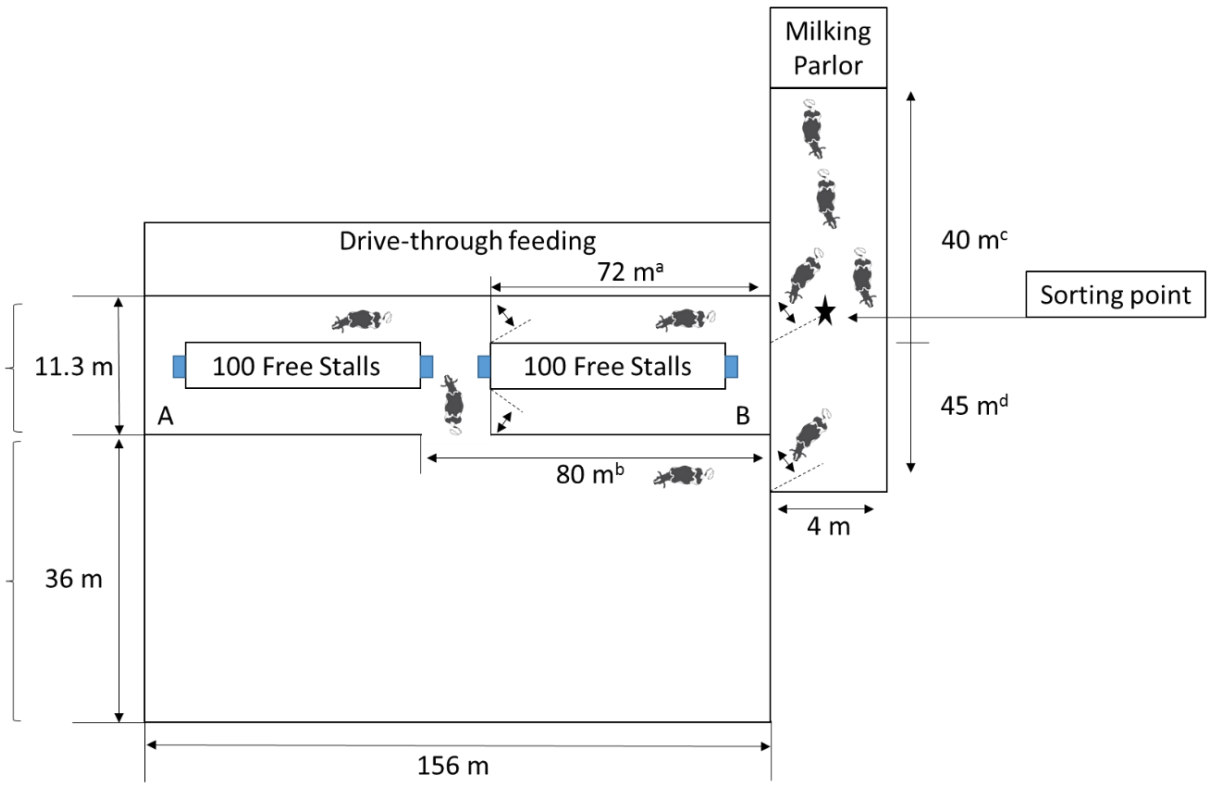


**Figure 1**. Layout of the research pen used for the RPF trial. Pen A housed the CON cows while pen B housed the ORG cows.

*Outcomes and data collection*

The response variables measured from the study cows included daily milk yield, biweekly milk components, resumption of ovarian cyclicity, pregnancy at 150 DIM, pregnancy per AI, pregnancy loss, body condition score (**BCS**), culling and mortality. All these variables were longitudinally measured from ORG and CON groups from calving until 150 DIM. Productivity was evaluated by daily milk yield, and by the accumulated milk yield at 21, 60 and 150 DIM. Individual daily milk yield (kg) were available from the farm records software (ALPRO, DeLaval, Tumba, Sweeden). Milk components were analyzed every other week through the DHI program. Components included fat, protein and lactose. Fat corrected milk (FCM = 0.4324*milk in lb. + 16.216*fat content) was calculated at every test day. Additionally, fluctuations in milk yield were evaluated before and after the grazing season, which started at 80 ± 11 DIM. This evaluation was standardized by DIM and the weekly milk yield averages were compared one week before grazing and for up to 5 weeks after grazing started.

Reproduction outcomes, such as cyclicity before 50 DIM were assessed through rectal ultrasonography. The presence of a corpus luteum (CL) was evaluated in two opportunities at 35 DIM and 49 DIM. If a CL was detected, the cow was determined as cycling. Farm personnel performed artificial insemination (**AI**) based on heat detection. Our research group evaluated pregnancy at 35 d AI. Pregnancy was confirmed 30 d later. Subsequently, we evaluated pregnancy loss 35 d after pregnancy confirmation. The number of artificial inseminations and the DIM at AI were obtained from farm records. Cyclicity and pregnancy were recorded as binary variables, whereas number of AI and DIM at AI were analyzed as count and time-to- event data. Blind evaluations of BCS were performed at 1, 3, 7, 21, 80 and 150 DIM using the standard scoring chart of 5 point with 0.25-point scale (Wildman et al., 1982). Additionally, BCS was assessed 7 d before

grazing season and 30, 50 and 75 d after grazing. Health data was evaluated as culling and mortality due to health reasons. These data were obtained from farm records.

One important aspect in nutritional supplementation trials is the calculation of the individual and group DMI. Nonetheless, as this was a nutritional trial performed in a commercial farm, there was no technical feasibility to measure DMI. To face this problem, the individual eating time (min/d) was estimated using accelerometers (CowManager SensOor, Agis Automatisering BV, Harmelen, the Netherlands) attached to the left ear (Pereira et al., 2018). The accelerometers are designed to differentiate spatial movements of the ear being associated to eating, rumination, and activity (walking-running) and could provide a reliable approximation of how much time the cows spent eating the treatment diets as well as their overall eating.

*Statistical Analysis*

Statistical software (SAS 9.4, SAS institute Inc., Cary, NC) was used for data analysis. Descriptive statistics for parity, DIM and univariate analysis were performed using Chi square test in SAS (PROC FREQ). Analyses of daily milk yield, average milk yield at 21, 60 and 150 DIM, milk components, FCM and BCS were performed using PROC MIXED for repeated measures. The evaluation of daily milk yield considered the sum of the three-daily milkings. The model included the fixed effects of treatment (ORG and CON) as fixed effects, parity (1; ≥2 lactation), DIM, and the interaction between treatment effect and DIM, while cow was considered a random effect. Average milk yield at 21, 60, 150 DIM and weekly milk yields during the grazing period were compared between treatment groups including treatment effect, parity and their interaction in the mixed model. Milk yield analyses during grazing included interaction terms between treatment group and week of evaluation, parity and week of evaluation, and a triple interaction term between treatment group, parity and week of evaluation. Milk fat and protein, and FCM were

compared by treatment group, parity and evaluation date, including the interaction between treatment group and evaluation date. For BCS treatment effects, parity and their interaction term were included.

The analysis of reproductive performance included cyclicity at 50 DIM, pregnancy at first AI and at 150 DIM, and pregnancy loss. These outcomes were analyzed through logistic regression (PROC LOGISTIC), including treatment group, parity and their interaction terms in the model. Additionally, to explore treatment effect on time to the first artificial insemination (AI) and pregnancy a survival analysis was performed (PROC LIFETEST). Wilcoxon *P*-values were used to test equality of strata (ORG and CON) of the survival curves.

Glucose, BHB, NEFA and BCS differences were examined using PROC MIXED for repeated measures, including treatment effect, sampling point, and treatment effect by sampling point interaction.

Overall health performance was evaluated by events of culling and mortality related to unspecific health disorders. Both culling and mortality were analyzed as binary outcomes. Simple logistic regression analyzed differences between treatment groups. Additionally, survival analysis evaluated time differences between groups for event of culling and mortality. Hazard ratios were calculated (PROC PHREG).

Differences in effective eating time between treatment groups were compared using PROC GLIMMIXED, by treatment groups and parity. The analysis was stratified between 0700 and 0800 h to compare eating time during the delivery of the treatments pellets. Additionally, overall, daily and weekly eating time were analyzed. The overall eating time model included treatment groups, parity, and their interaction, whereas weekly eating time included treatment group, week in milk,

and their interaction. The daily eating time model included treatment group and DIM, and their interaction.

Statistical significance was determined at *P*-values < 0.05. LSM differences were analyzed using the Tukey-Kramer test. Variables with *P*-values ≤ 0.15 were kept in the models for confounding control.

**Results and Discussion**

To support the inclusion of an organic certified RPF in diets of organic lactating dairy cows, the supplementation of RPF should show improvements on health and reproductive performance, as well as on productivity. All these outcomes are closely related to overcoming the metabolic syndromes related to exacerbated tissue mobilization and energy balance hat most cows undergo during the first 60 days of lactation.

*Productive performance*

The effects on milk yield and milk components were assessed after the inclusion of ORG in the diets of early lactating dairy cows from calving until the mid-lactation stage. The analysis of the daily weights as a continuous outcome showed greater milk production in ORG cows. Cows from ORG group produced 1.6 kg/d more milk compared to control cows (32.1 ± 0.57 vs. 30.5 ± 0.6 kg/d; $P = 0.04$) up to 150 DIM. Additionally, parity and DIM showed statistical significance in the model ($P < 0.0001$). The interaction term between treatment groups and DIM had a significant effect ($P = 0.013$) when estimating differences between the study groups (ORG vs. CON). Although the significant interaction term should be reported as the main result of the daily milk yield analysis, its complexity for a correct interpretation during prolonged observations periods makes necessary the observation of the main effects of treatment through the analysis of the plotted milk curves over time. Figure 2 shows a clear deviation on the LSM of milk yield

during specific periods. During the first 21 DIM the ORG group had greater milk production. Between 21 DIM and 50 DIM, ORG and CON cows had similar production, which may explain the statistical significance of the interaction term. The ORG group maintained greater milk yield until the end of the observation period.

Other studies on RPF have shown contradictory results on milk yield when supplemented in conventional dairy systems. On one hand, under conventional management, some studies have reported a positive effect on milk yield. NcNamara et al. (2003) tested the effect on milk yield after the supplementation of two commercial RPF, for 134 d, using a similar dose to that used in this study (0.45 kg/d). One RPF (Megalac Plus®) increased milk yield by 1.5 kg/d up to 12 weeks of lactation compared to the control group, whereas the study did not detect differences for the other RPF (Megapro Gold®). Hammon et al. (2008) determined that cows fed with RPF after a corn starch diet tended to produce 1.8 kg/d more milk compared to control at mid-lactation stage from 80 to 110 DIM ($P = 0.05$). These differences agree with what is depicted in the plot showed in Figure 2, where daily milk weights started to separate between groups and remained different until the end of the follow-up period.

On the other hand, other studies did not detect improvements on milk yield when supplementing RPF. Lohrenz et al. (2010) investigated the inclusion of RPF (N = 18) in mid lactation cows (98 DIM) for 4 weeks. Under these study settings, the researchers did not find differences in daily and weekly milk yield, with both groups producing approximately 32.7 kg/d. These results contrast with those found in this study during the mild lactation stage. Although, the management differs due to grazing and organic production, in our study it was determined that ORG cows produced more milk after 90 DIM (Figure 2).

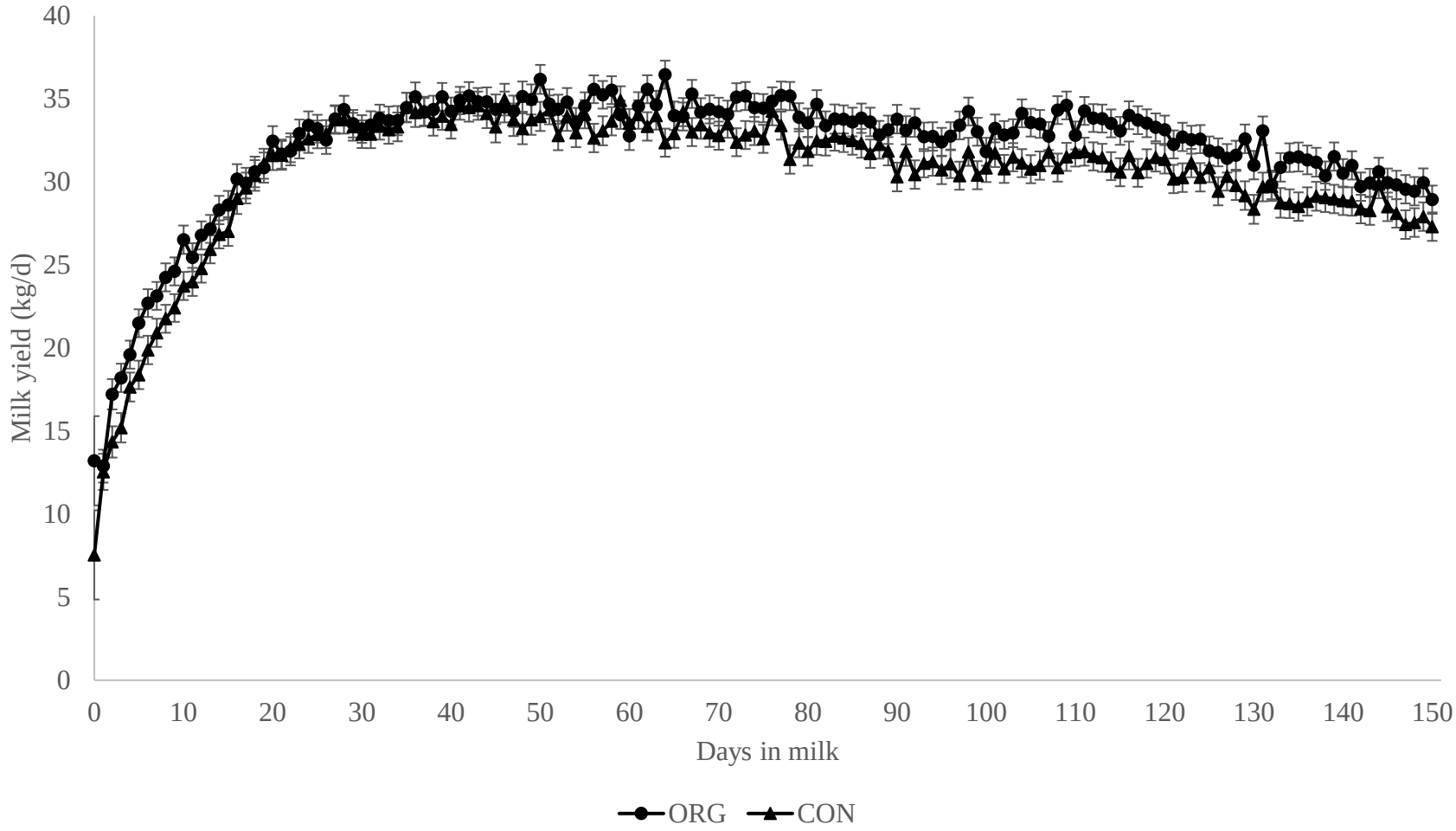


**Figure 2.** Comparison of the lactation curves between dairy cows supplemented with organic rumen protected fat (ORG) and control cows (CON) between 1 and 150 DIM. Interaction treatment by DIM: $P = 0.013$

Another study by Kitessa et al. (2004), supplemented post-partum dairy cows (N = 14) with RPF tuna oil during the grazing season. Although, the objective of that study was to investigate the effect of tuna oils on sensory characteristics of milk, the extra energy provided by the RPF did not increase the milk yield.

Under intensive management systems, frequent animal movements between hospitals and lactating groups are common. This makes sample sizes of research pens to variate day by day. For this reason, the overall milk yield in specific periods, which are particularly challenging for dairy cows, could assist in the analysis of dietary interventions offering a more intuitive information for organic dairy farmers. However, this approach does not include the information of daily milk yield fluctuations within the treatment groups, as well as the auto-correlative nature of the data. The analysis of milk yield averages (LSM) up to 21 DIM showed a significant effect of the supplemented diets. The group fed with ORG produced 1.1 kg/d more than the CON group (25.5 ± 0.26 vs. 24.4 ± 0.25 kg/d; $P = 0.003$). The effect of parity was also significant ($P < 0.0001$) but no interaction was detected ($P = 0.11$).

The level of milk production after the fresh period could reflect the energetic efficiency of a group of cows (Jenkins and McGuire, 2006), where cows receiving a more concentrated source of energy, such as FA, can produce more milk (Vasquez-Añon et al., 1997). The average milk produced at 60 DIM showed a significant interaction between treatment group and parity ($P <0.0001$), where cows (lactation number ≥ 2) fed with ORG produced 1.13 kg/d more milk than CON cows (36.7 ± 0.15 vs. 35.4 ± 0.15 kg/d; $P < 0.0001$). On the other hand, there was no difference between heifers in both treatment groups ($P = 0.43$). The analysis of the total average milk at 150 DIM indicated an interaction between treatment group and parity ($P <0.0001$).

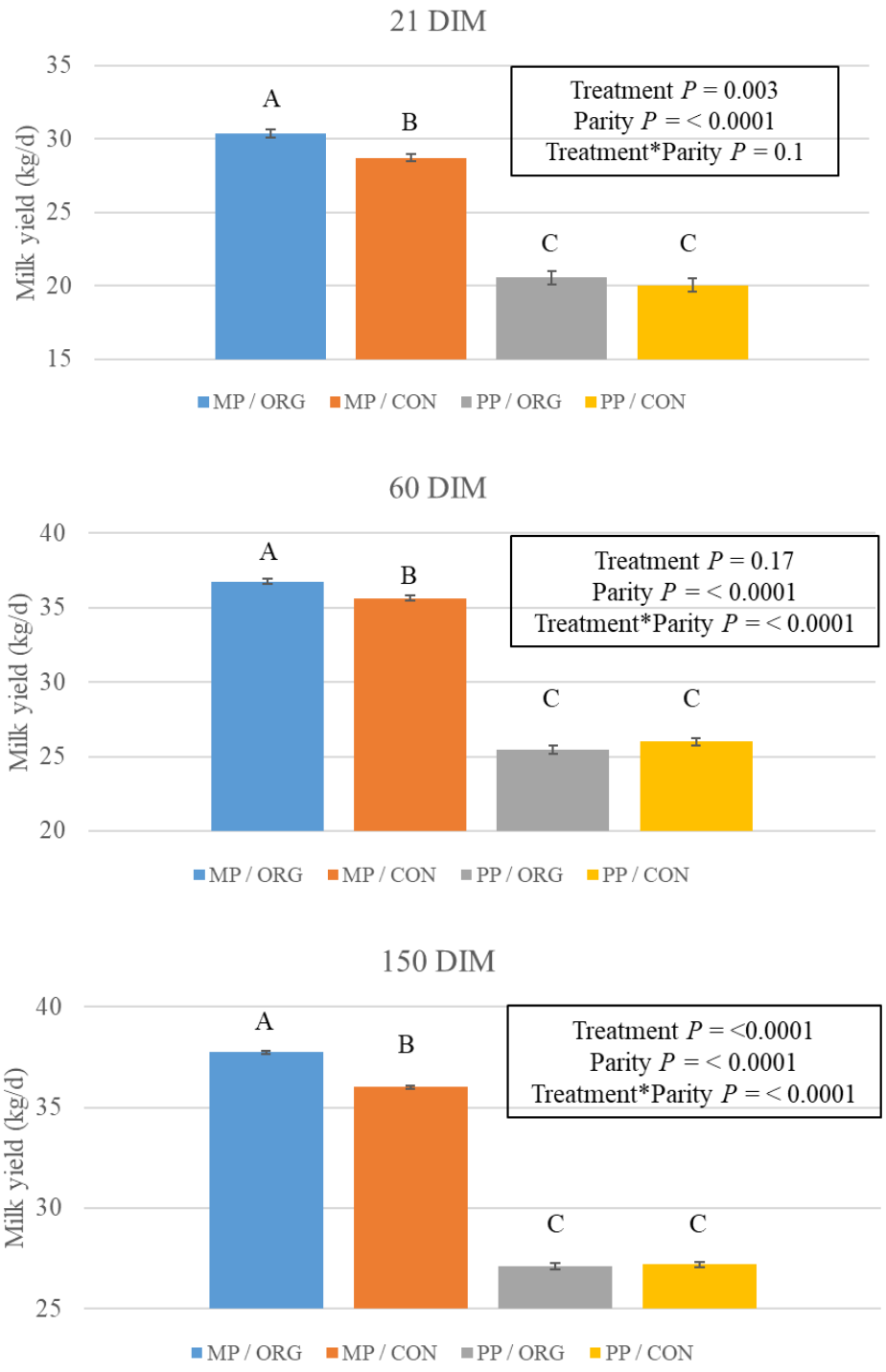


**Figure 3.** Least square means and SE of milk yield at 21, 60 and 150 DIM per ORG and CON groups and parity (MP: multiparous; PP: primiparous). Different letters indicate statistical differences at $P = 0.05$.

The production of mature cows under the ORG supplementation was 1.7 kg/d higher compared to CON cows (37.7 ± 0.09 vs. 36 ± 0.09 kg/d; $P$ <0.0001). Milk yield at 21, 60 and 150 DIM by treatment group is presented in Figure 3.

As depicted in Figure 3, there was no difference for milk yield between heifers from both treatment groups. Although it is known that MP produce more milk than PP cows due to PP's higher energy requirements for growth, milk production and an underdeveloped mammary gland (Grummer, 1995), we expected greater milk yield on ORG/PP cows. Other studies on FA supplementation had similar results on heifers, where differences in milk yield were observed in MP but not in PP cows (Souza and Lock, 2018, Holter et al., 1992). This may be explained for greater energy requirements and mammary gland development (Grummer, 1995), and for differential eating behavior between MP and PP cows, where PP cows have lower DMI but visit the feeder more frequently and by a shorter time (Neave et al., 2017). The last case might explain the results observed in this study because the treatment pellets were delivered once a day by a restricted period, which could have limited the access of heifers to the pellets, although the same conditions affected both treatment groups.

There are inconsistent results on the responses of milk yield and milk solids across published studies on RPF and FA supplementations and the effects of this different dietary energy sources are poorly understood. Contradictory results in the literature might be explained by different study settings, sample sizes, intake of the treatment diets, and productive potential of the animals. In this study, we observed consistent increases in daily milk weights during almost all lactation stages. However, the next questions that arise is how the energy source provided by the organic protected fat was used to overcome NEB and whether the FA in the pellets improved the glucose availability for the mammary gland instead of being used for maintenance.

Energy status affects the mammary gland metabolism (Hammon et al., 2008). Thus, changes in lactose, milk protein and fat have been reported when supplementing RPF (Hammon et al., 2008; Duske et al., 2009; Lohrenz et al., 2010).

The responses on productive performance due to the input of concentrated energy in the diet can be assessed not only through the milk weights but also in milk components such as fat and proteins, which are of interest to dairy farmers. Changes in milk fat may be affected in a greater extent by dietary interventions compared to the protein content, which is putative to the genetic component of the cow with genetic covariances between 33 to 79% (Morton et al., 2018). During this study, milk components were tested for a total of seven times. However, at sampling day there was variation in DIM among the study cows (26 d difference between the first and last enrolled cow). For this reason, we stratified the analysis of milk components by DIM in four evaluation times so that provided each cow had the chance to be tested at least twice in each evaluation time. A summary of milk components by study group is presented in Table 2. No differences between treatment groups were found in the number of cows sampled per evaluation time ($P = 0.9$). Additionally, the interaction between treatment group and evaluation time was not significant in the analysis of fat and protein ($P = 0.9$) and therefore this term was removed from the models. The final model included treatment effect, parity and evaluation time. Milk fat content did not differ between ORG and CON cows (3.86 ± 0.03 vs. 3.92 ± 0.03%; $P = 0.16$). Mature cows had higher fat content compared to first lactation cows (3.95 ± 0.02 vs. 3.83 ± 0.04%; $P = 0.013$). Additionally, there was a significant effect of evaluation time in the milk fat content ($P < 0.0001$), where fat content was decreasing from early lactation stages until the last sampling. In the analysis performed before 30 DIM, milk fat averaged 4.4% whereas samples collected after 100 DIM averaged 3.6%.

**Table 2.** Milk fat, protein and 3.5% fat corrected milk (FCM) comparison between cows supplemented with organic rumen protected fat (ORG) and control cows (CON).

| Evaluation | Variable | ORG | CON | Difference | P-value |
|---|---|---|---|---|---|
| 1 | Fat (%) | 4.37 | 4.39 | -0.02 | 0.83 |
| | Protein (%) | 2.96 | 3.03 | -0.07 | 0.053 |
| | FCM (kg) | 43.35 | 43.30 | 0.05 | 0.68 |
| 2 | Fat (%) | 3.83 | 3.88 | -0.05 | 0.58 |
| | Protein (%) | 2.62 | 2.63 | -0.01 | 0.9 |
| | FCM (kg) | 42.78 | 42.98 | -0.19 | 0.8 |
| 3 | Fat (%) | 3.61 | 3.68 | -0.07 | 0.4 |
| | Protein (%) | 2.64 | 2.62 | 0.02 | 0.67 |
| | FCM (kg) | 40.71 | 41.08 | -0.36 | 0.62 |
| 4 | Fat (%) | 3.62 | 3.73 | -0.11 | 0.23 |
| | Protein (%) | 2.72 | 2.70 | 0.02 | 0.34 |
| | FCM (kg) | 41.18 | 41.13 | 0.06 | 0.94 |

Evaluation time 1: samples collected between 1 – 30 DIM; Evaluation time 2: samples collected between 31 – 50 DIM; Evaluation time 3: samples collected form 51 – 100 DIM; Evaluation time 2: samples collected between 101 – 150 DIM.

Hammon et al. (2008) observed that cows supplemented with RPF tended to decrease milk fat ($P = 0.08$). However, another study assessing commercial RPF has found no differences in milk fat (NcNamara et al., 2003) when using similar supplementing amounts to our study. Rumen-protected fats from different sources have also been evaluated regarding milk components. Soybean and tuna oil RPF have shown no differences in milk fat after supplementation (Kitessa et al., 2004; Lohrenz 2010; Pappritz et al., 2011). However, Duske et al., (2009) suggested that the differences in milk fat should be observed on the milk FA profile, especially in unsaturated FA (Palmitoleic acid) that tend to increase with the use of RPF.

Milk protein had the same pattern across treatment groups and evaluation dates. Overall, milk protein did not differ between ORG and CON cows (2.74 ± 0.01 vs. 2.76 ± 0.01%; $P = 0.17$). Parity had a significant effect on milk protein, with first lactation cows having higher protein content compared to mature cows (2.77 ± 0.02 vs. 2.73 ± 0.01; $P = 0.039$). Most studies have concluded that RPF did not alter milk protein percentage (Kitessa et al., 2004; Hammon et al., 2008; Duske et al., 2009; Lohrenz et al., 2010). Conversely, NcNamara et al. (2003) concluded that supplementation of commercial RPF reduced milk protein. This study had very similar settings to our study (201 cows including PP and MP) and reached similar conclusions regarding fat and protein, our results were close to tendency showing that ORG decreased milk protein.

No difference in the overall 3.5% FCM between treatment groups (ORG = 42.12 ± 0.25 vs. CON 42.1 ± 0.26 kg/d; $P = 0.74$) was determined in our study, whereas parity and evaluation time had statistical significance. Multiparous cows had greater FCM production than primiparous cows (44.6 ± 0.2 vs. 39.5 ± 0.33 kg/d; $P < 0.0001$). FCM is used as a measure of dietary energy and efficiency of the dairy, which is of interest for dairy farmers (Britt et al., 2003).

Nonetheless, few studies on fat supplementation have analyzed FCM. Among those, Hammon et al. (2008) and Lohrenz et al. (2010) did not find a significant increment on FCM when supplementing RPF in lactating dairy cows.

To this point, the main effect of the organic RPF tested in this study was higher milk yield. However, other factors that may affect milk yield should be controlled. We made efforts in reducing selection bias by blocking and randomizing the study animals according to their parity and previous lactation productivity between ORG and CON groups ($P = 0.22$). Nonetheless, other issues during the implementation of the trial may have affected the ability to accurately attribute an effect to the organic RPF. One factor to consider is the number of cows with dry quarters that, by chance, might affect milk yield of one treatment group. As in organic dairy farming, the use of antimicrobial therapy for mastitis is banned, a common practice to treat intramammary infection is to strip the affected quarter for a couple of days and eventually stop its milking. For this reason, a retrospective analysis was performed to examine whether there was an unbalanced proportion of cows with dry quarters between groups, and whether there was an interaction between dry quarter proportion per group and milk yield at 150 DIM. Dry quarter data was collected from farm records. The proportion of cows with dry quarters did not differ between ORG and the CON group (19% vs. 14%; $P = 0.49$). To investigate the confounding magnitude of dry quarters on the average of the daily milk yield up to 150 DIM, a mixed model was used including treatment group, parity, presence of dry quarters (as binary variable), and the interaction between treatment group, parity and treatment group and dry quarter. The presence of dry quarters and parity interacted with the treatment group ($P < 0.0001$ and $P = 0.002$, respectively). Interestingly, cows with dry quarters seem to compensate their milk production and produce more milk in comparison to cows with four

functional quarters (33 ± 0.14 vs. 31.5 ± 0.06 kg/d; $P < 0.0001$). These differences were also observed when comparing the effect of the inclusion of the organic RPF (Figure 4).

Treatment cows affected with dry quarters produced 1.8 kg/d more compared to CON cows with all functional quarters at 150 DIM (32.6 ± 0.18 vs. 31.1 ± 0.1 kg/d; $P < 0.0001$). On the other hand, when comparing treatment groups affected by dry quarters the effect of ORG was diluted by the milk increase compensation in both treatment groups (Figure 4). Therefore, the ORG group with dry quarters produced 32.9 ± 0.18 kg/d at 150 DIM, whereas the CON group with dry quarters produced 33.1 ± 0.3 kg/d ($P = 0.25$).

Although the stratification by dry quarter partitions the sample size and it may reduce the power to detect differences in the effect of ORG, the study of nutritional supplementation on cows with specific conditions could represent a good approach for differential intervention and feeding strategies. Additionally, milk yield analysis was restricted to the grazing season to evaluate the performance of cows supplemented with the RPF. Certified organic dairy farms are required to graze their cows at least 120 days per year (NOP, 2013). To obtain a comparison baseline, daily milk yield average for the 7-d before grazing was compared between treatment groups. Average DIM at the start of grazing was 83.5 ± 6.3, and weekly comparisons were performed. Mature cows eating the RPF produced more milk the week before grazing started and the following five weeks; however, these differences were not observed between primiparous cows (Figure 5), similarly to the results in the overall productive performance.

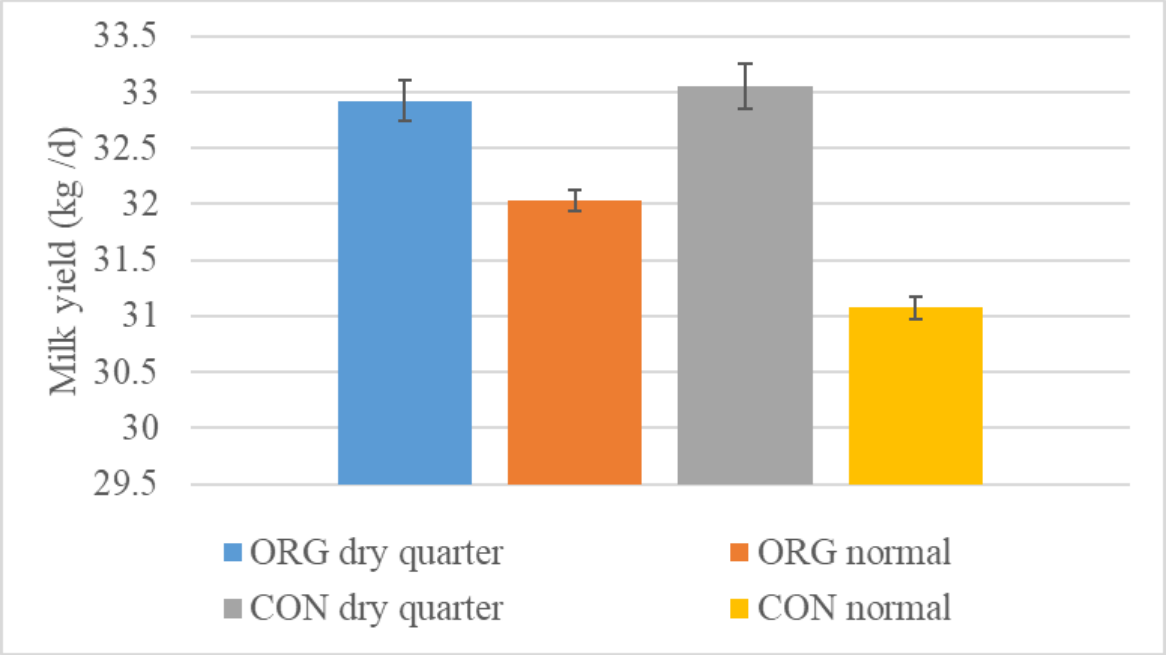


**Figure 4.** Stratified milk yields at 150 DIM by presence of dry quarters in cows supplemented with organic rumen protected fat (ORG) and control (CON).

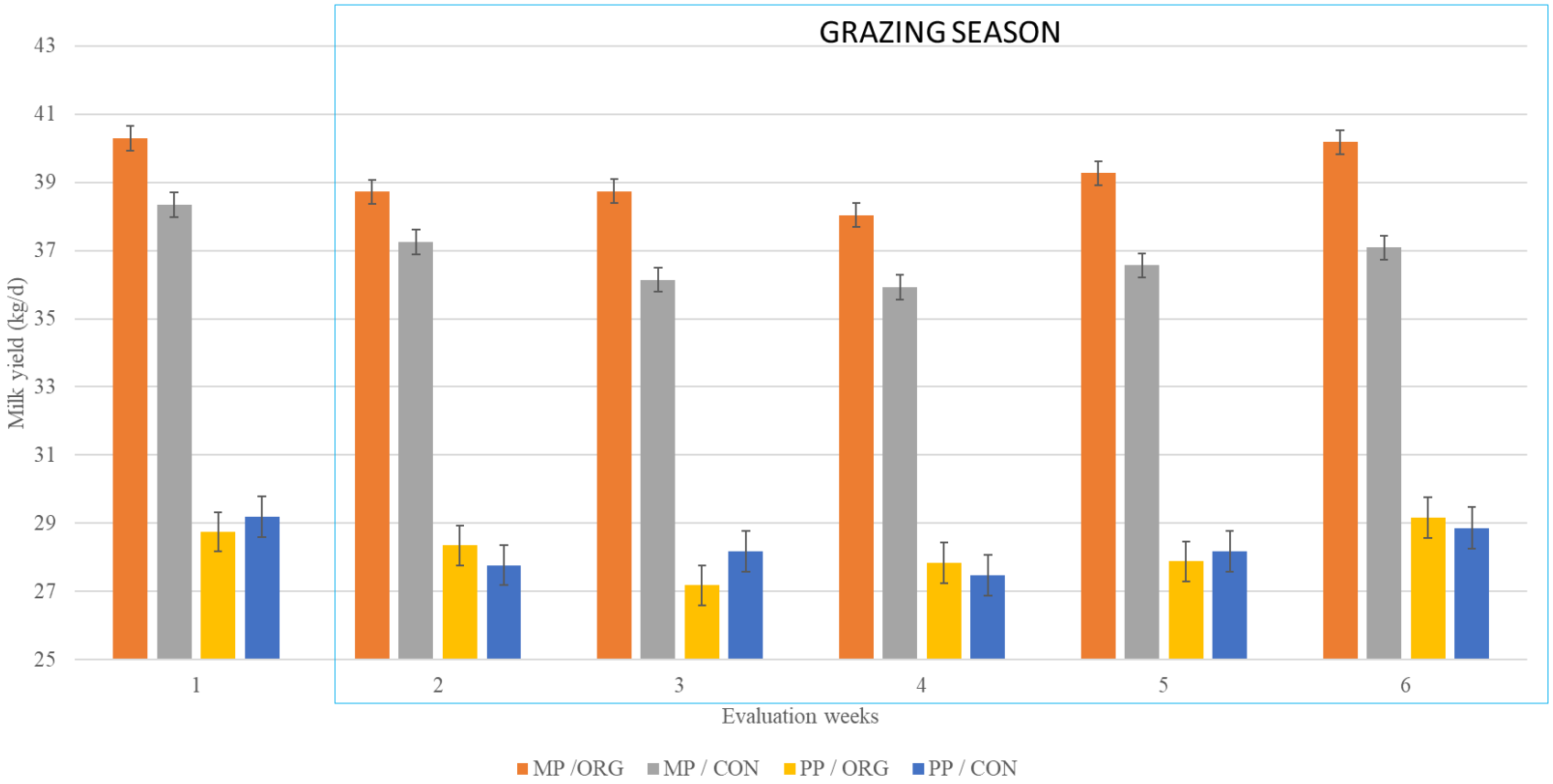


**Figure 5.** Weekly LSM (and SE bars) of milk yield in cows supplemented with organic rumen-protected fat (ORG) and control (CON) cows during the week prior (1) and during the grazing season (MP: multiparous; PP: primiparous).

*Reproductive performance*

The effects of dietary interventions during transition are complex and multifactorial (Rodney et al., 2018). Several nutritional interventions on transitioning dairy cows have investigated pregnancy rates, resumption of cyclicity, calving interval, and number of AI per pregnancy as measures of reproductive performance. However, it is very difficult to attain greater reproductive efficiency through a single nutritional management as most strategies are focused into increasing the energy and nutrient availability but their interaction with physiological pathways is usually unknown and the outcomes are limited to binary responses.

Nonetheless, it has been recognized that some nutrients improve reproductive performance. Rodney et al. (2018) suggested that increased FA, starch, and metabolizable energy balance intake was positively associated with the proportion of pregnant cows. On the other hand, the authors concluded that the increased intake of rapidly fermentable sugars and high milk protein yield are associated with reduced proportion of pregnant cows. Unfortunately, inconsistencies in the study designs and low sample sizes when analyzing binary outcomes, limit the validity of the conclusions about the effect of nutritional interventions in dairy cattle (Lean et al., 2016). In our study, we supplemented organic rumen-protected FA (Table 3) during the first 150 DIM. Therefore, our results will be contrasted with other studies using FA as energy source. To this point, this study and others have advocated the positive results on milk yield without deleterious effects on milk components to the energy input provided by RPF.

Negative EB in dairy cows is associated with reductions on LH pulse frequency, growth rate and diameter of dominant follicle, weight of the corpus luteum estradiol and progesterone (Pryce et al., 2004; Van Knegsel et al., 2005). Besides the increment of energy density, polyunsaturated FA influence fertility in farm animals by modulating the biosynthesis of

prostaglandins, steroids and transcriptional regulation of genes involved in the control of fertility (Waters et al., 2012; Marei et al., 2018). The FA content of the organic RPF used in this study was formulated to match the FA profile of the RPF available in the marketplace; therefore, similar effects regarding reproductive performance could be expected.

Overall, there was no significant improvement on the reproductive responses evaluated in this study. There was no significant interaction between treatment effect and parity for cyclicity at 60 DIM and pregnancy at 150 DIM so this term was removed from the models. Resumption of ovarian cyclicity at 50 DIM was not associated with the treatment diets ($P = 0.81$). To the best of our knowledge, this is the first report of the effects of RPF on cyclicity in organic dairy cows. Parity had a significant effect, where MP cows had greater odds of returning to ovarian cyclicity compared to PP animals (OR [95% C.I] = 2.03 [1.04 – 3.9]; $P = 0.039$). Seventy-seven animals resulted pregnant at 150 DIM. However, there were no differences between treatment group and parity according to the logistic regression models ($P = 0.4$ and $P = 0.9$, respectively). The number of cows that resulted pregnant at first AI did not differ between treatment groups ($P = 0.43$). Nine cows had pregnancy loss during the first 60 of gestation but no differences were found between treatment groups ($P = 0.4$). Few studies on RPF have investigated reproductive performance, McNamara et al. (2003) reported no differences in the conception rate at the first AI in dairy cows supplemented with conventional RPF compared to CON cows. In agreement, we did not find differences in the same outcome ($P = 0.99$). Additionally, McNamara et al. (2003) considered conception rate to second AI, where they found that cows supplemented with RPF had greater conception rates. Conversely, we did not find differences in this outcome despite both studies having similar sample size (nearly 200 cows). Finally, no differences were found in the number of services per pregnancy in both studies.

**Table 3.** Fatty acid profile of the supplemented organic rumen-protected fat (RPF [Organilac]) and a conventional RPF

| Fatty acid | Organilac (%) | Conventional RPF (%) |
|---|---|---|
| Mistiric | 0.9 | 0.5 |
| Palmitic | 45 | 46.2 |
| Stearic | 3.9 | 2.6 |
| Oleic | 39.3 | 41 |
| Linoleic | 9.3 | 9.4 |
| linoleinic | 0.3 | 0.3 |

Source: Organilac, product information sheet (Organic Animal Nutrition, 2018)

Although we did not find differences in the proportion of cows pregnant by treatment groups, it is interesting to explore whether there were temporal differences in pregnancy rates. Survival curves were obtained to analyze the probability rates of pregnancy over time. Even though the survival curve of ORG cows was under the CON cows' curve (Figure 6), the survival functions did not differ between groups ($P = 0.4$).

*Blood metabolites, BCS, culling and mortality*

Negative EB is usually evaluated through some metabolites reflecting the cow's adaptation to transition, reproduction and the risk of peri-partum diseases and culling (Melendez et al., 2006; Duffield et al., 2009; Melendez et al., 2009; Abdelli et al., 2017; Ruprechter et al., 2018). High levels of NEFA have been associated with high risk of LDA, clinical mastitis and milk fiver (LeBlanc et al., 2005; Melendez et al., 2009), whereas high BHB levels are associated to greater risk of SCK, metritis, mastitis, decreased DMI, milk yield and NEB (LeBlanc et al., 2005; Duffield et al., 2009; Overton et al., 2017). Additionally, Duffield et al. (2009) concluded that BHB levels $\geq$ 1.2 mmol/L increased milk fat. Thus, NEFA and BHB are typically evaluated to measure underlying NEB (Overton et al., 2017). On the other hand, carbohydrate metabolism markers are not as well investigated as lipid metabolites in dairy cows. However, research in this may contribute to a better understanding of the insulin resistance that most transition Holstein dairy cattle undergo in intensive dairy farming in the US (De Koster and Opsomer, 2013). Carbohydrate metabolism in ruminants is characterized by low circulating levels of glucose with a high demand by the mammary gland during lactation (0.4 mol/Kg of milk) that conditions high milk production in dairy cows (Lohrenz et al., 2010). All glucose metabolism is governed by different hormones, in which insulin plays a key role.

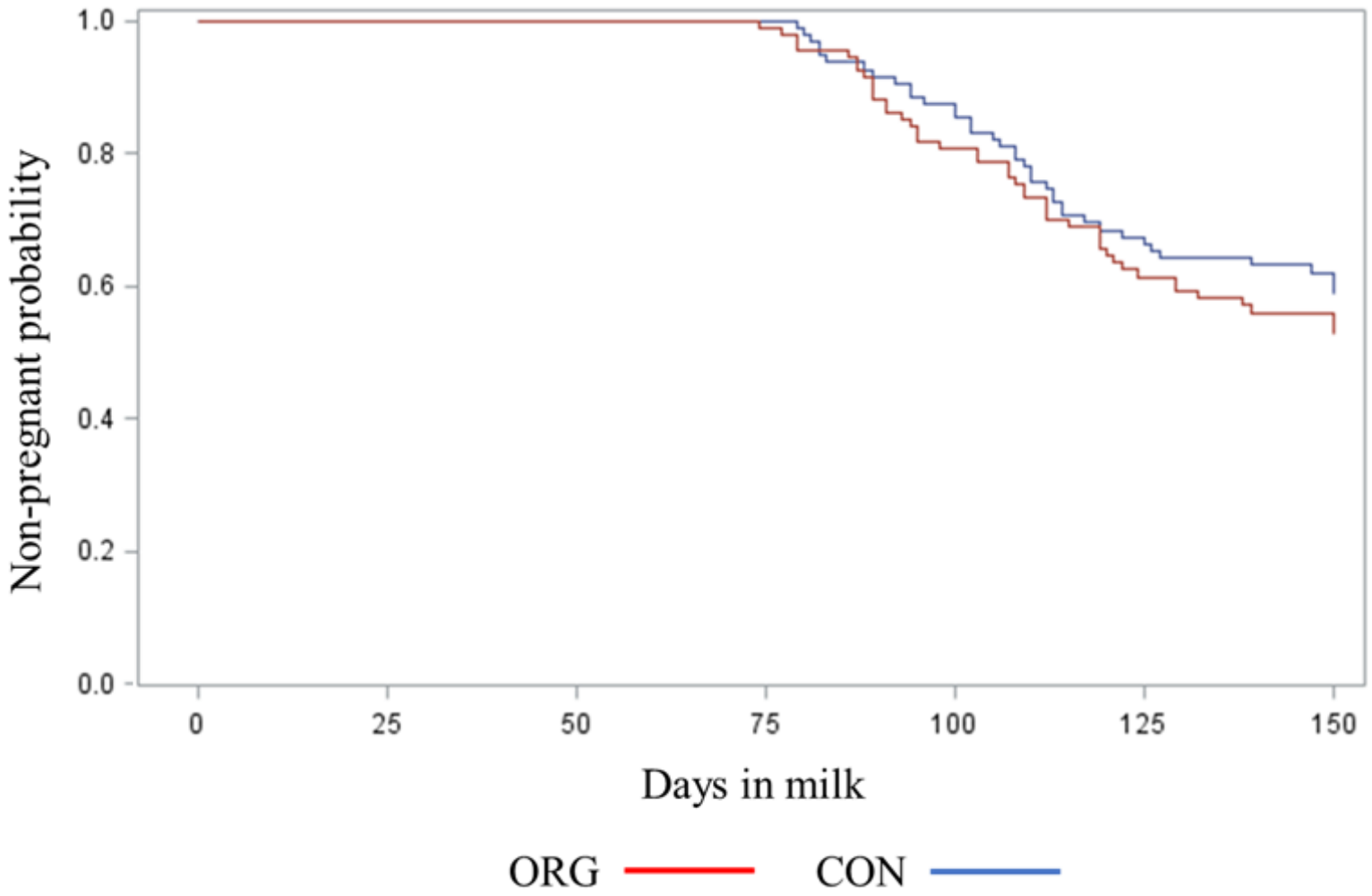


**Figure 6.** Survival curve of pregnancy probabilities up to 150 DIM from the dairy cows supplemented with organic protected fat (ORG, red line) and control (CON, blue line).

Insulin levels control several gluconeogenic glycolytic pathways where lipolysis, amino acid, skeletal muscle and ruminal fermentation (production of VFA as major precursor of hepatic gluconeogenesis) are involved (De Koster and Opsomer, 2013). This complex metabolic and endocrine interaction limits a whole understanding of the effects of dietary supplements after calving when few metabolites are analyzed (Overton et al., 2017; Ruprechter et al., 2018). Nonetheless, some studies have described the relationships of serum metabolites and some productive traits in dairy cattle. For example, there is a positive correlation between BHB and NEFA and a negative correlation between BHB, NEB and glucose (Overton et al., 2017). This might be used to infer the adaptive responses that are being facilitated by feed additives in controlled trials.

Dairy cows have adaptive responses after calving to satisfy the increasing glucose requirements for lactation. The main adaptation against NEB consists in shifting to a lipogenic metabolism, where ketone bodies and free FA are the main source of energy (Herdt, 2000). Therefore, increased levels of BHB and NEFA besides decreased insulin sensitivity might be expected in post-partum dairy cows. However, poor DMI and insufficient glucose supply elicit excise fat and muscular tissue mobilization and excessive accumulation of ketone bodies and NEFA favoring a pathologic state of hyperketonemia (Herdt, 2000; Duffield et al., 2009).

Rumen-protected fat addition in rations of lactating dairy cows aims to increase the energy input during early post-partum. This lipogenic diets are recognized to increase peripheral NEFA and BHB to be used as primary source of energy and to reduce serum glucose (Van Knegsel et al. 2005). We analyzed serum glucose, BHB and NEFA at 1, 3, 7 and 21 DIM. Serum levels of those metabolites are presented in table 4. There was no significant treatment effect on serum glucose and BHB. Hammon et al. (2008) and Lohrenz et al. (2010) did not observe differences in glucose

but cows fed with RPF tended to have lower glucose concentrations ($P$ = 0.1). Accordingly, we did not find differences in glucose between treatment groups across sampling points (Table 4 and Figure 7,b).

Regarding BHB serum concentrations we observed a tendency in the main treatment effect ($P$ = 0.11), consequently, the plot of BHB fluctuations shows greater BHB levels in ORG cows (Figure 7). This agrees with the results reached by Hammon et al. (2008), Lohrenz et al. 2010 and Pappritz et al. (2011) where RPF were fed post-partum. On the other hand, when RPF is fed in the last trimester of lactation BHB has been observed to decrease in the subsequent lactation (Duske et al., 2009).

Non-esterified fatty acids levels are usually analyzed to assess the adaptation of cows to transition (Drackley, 1999) and they are considered in evaluating the effectiveness of RPF on improving the energy balance. Most studies have found no statistical differences on NEFA serum concentration after RPF supplementation. However, most studies presented tendencies for RPF tending to increase NEFA concentrations, suggesting that RPF increases circulating BHB and NEFA (Hammon et al., 2008; Duske et al., 2009; Lohrenz et al., 2010; Pappritz et al., 2011). This may be explained because lipogenic precursors elicit a surplus of lipid metabolites to be used as energy source (Van Knegsel et al. 2005) and in the β-oxidation of FA (Hammon et al., 2008). Our results agree with this evidence as feeding organic RPF tended to increase NEFA concentrations (Figure 7,c; Table 4).

**Table 4.** Glucose, β-Hydroxybutyrate (BHB) and Non-esterified fatty acids (NEFA) serum levels in cows supplemented with organic rumen-protected fat (ORG; n = 54) and control cows (CON; n = 58)

| | Glucose mg/dL | | | BHB mmol/L | | | NEFA mEq/L | | |
|---|---|---|---|---|---|---|---|---|---|
| Days in milk | ORG | CON | *P* -value | ORG | CON | *P* -value | ORG | CON | *P* -value |
| 1 | 94.08 | 88.9 | 0.23 | 1.3 | 1.23 | 0.72 | 0.36 | 0.34 | 0.19 |
| 3 | 60.51 | 57.2 | 0.45 | 1.8 | 1.6 | 0.33 | 0.26 | 0.22 | 0.008 |
| 7 | 59.4 | 56.7 | 0.53 | 1.7 | 1.3 | 0.06 | 0.23 | 0.19 | 0.04 |
| 21 | 58.4 | 58.5 | 0.98 | 1.53 | 1.23 | 0.14 | 0.17 | 0.13 | 0.06 |

Nonetheless, regarding BHB concentrations, 35% of sampled cows were above the cut-off value for subclinical ketosis (> 1.2 mmol/L), within both treatment groups. As shown in Figure 7b, ORG cows had higher mean concentration of BHB at 7 and 21 DIM over 1.2 mmol/L. Despite these results, ORG cows still showed more milk yield and lower body condition loss during the first 80 DIM. As previously discussed, we might attribute higher BHB concentration to the higher availability of free FA provided by the organic RPF, which could favor higher rate of β-oxidation of FA. Nonetheless, BHB group means in control group were also above 1.2 mmol/L. This condition may be explained by particular circumstances of organic management favoring the presence of outliers in the tested animals or to lack of accuracy in the BHB stripe test used in this study.

Although high BHB and NEFA levels and low glucose and insulin levels during the first three weeks post-partum are associated with NEB, poor productive, and reproductive performance and metabolic diseases (Van Knegsel et al., 2005; Melendez et al., 2009; Abdelli et al., 2017; Overton et al., 2017), such fluctuations are normal in periparturient adaptation. Thus, the pathological status of those changes induced by the addition of dietary supplements should be determined observing other factors such as BCS and health.

Body condition score changes during transition have been associated with milk yield, post-partum health and decreased fertility (Carvalho et al 2014; Bedere et al., 2018; Ruprechter et al., 2018). Therefore, these parameters should be included in the assessment of transition success and metabolic responses to different transition management strategies. Despite its importance, few studies on RPF supplementation have included BCS as a response variable.

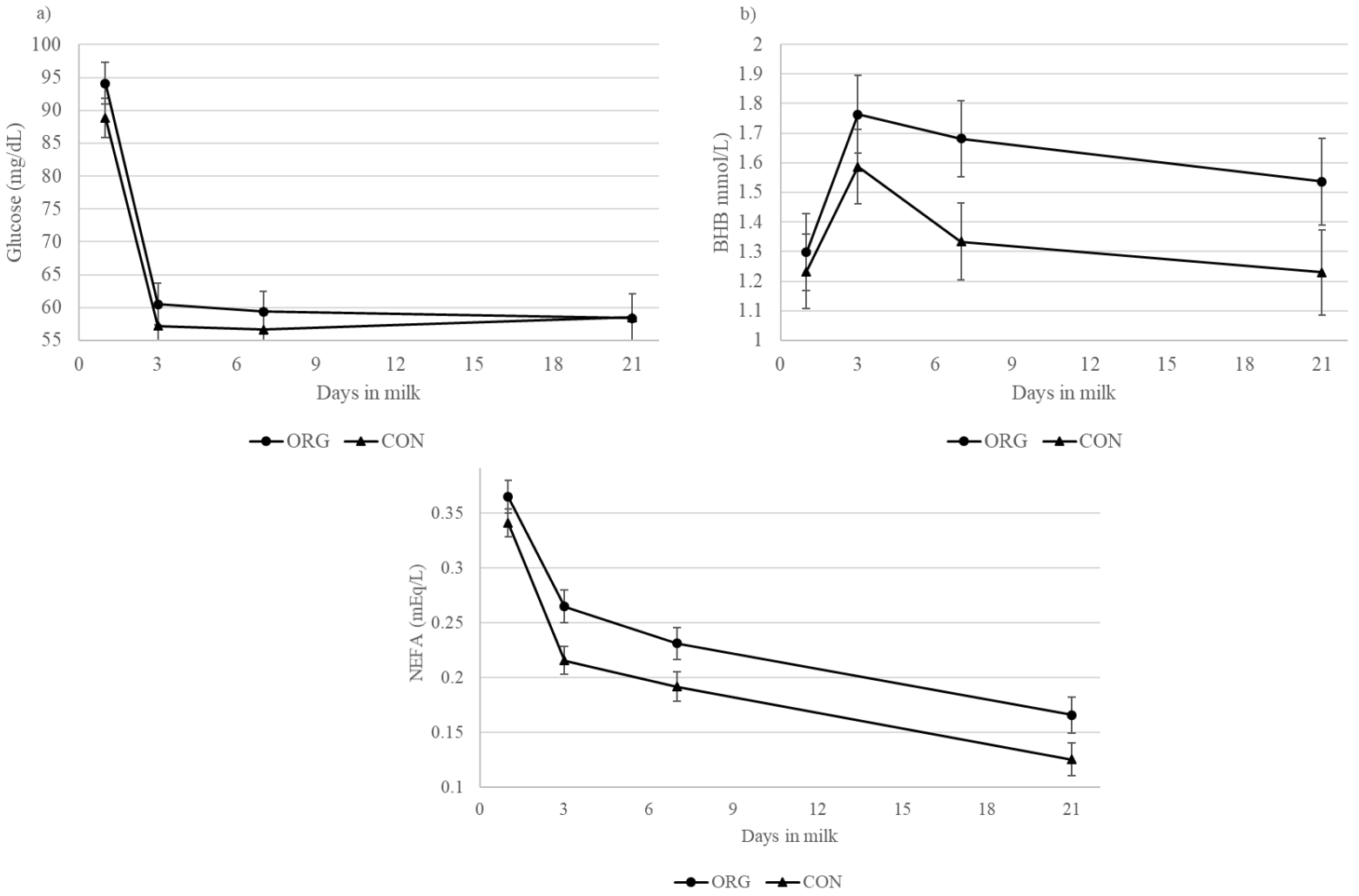


**Figure 7.** Glucose (a), β-Hydroxybutyrate (BHB; b) and non-esterified fatty acids (NEFA; c) serum concentrations at 1, 3, 7 and 21 DIM in cows fed with organic rumen-protected fat (ORG; n = 54) and control diets (CON; n = 58)

We evaluated BCS as a measure of lipid and protein tissue mobilization in response to a major availability of FA absorbed in the small intestine. This is related to the fact that if energy expenditure exceeds the energy intake the cows will lose weight (Van Knegsel et al., 2005). Before the beginning of supplementation with the organic RPF, the study cows were BC scored within 24 h post-partum. There was no association between treatment assignment and BCS at that point ($P$ = 0.8). Overall, MP cows tended to have greater BCS compared to PP cows at 1 DIM ($P$ = 0.06). BCS responses at different evaluation times are presented in Table 5 and Figure 8. There were no differences at 1 and 3 DIM between ORG and CON cows. There was a treatment effect in the evaluations performed at 7 (2.91 ± 0.03 vs. 2,8 ± 0.03 BCS points; $P$ = 0.02), 21(2.85 ± 0.04 vs. 2.75 ± 0.04 BCS points; $P$ = 0.04) and 80 DIM (3.1 ± 0.04 vs. 2.9 ± 0.04 BCS points; $P$ = 0.0005) where ORG cows had greater BCS and had lost body condition in a lower extent up to 21 DIM (Figure 8). These differences may be associated with the milk yield at these periods (Figure 2), since ORG cows had greater production along with lower loss of body condition, which could be attributed to the greater energy input delivered by the organic RPF. Pappritz et al. (2011) evaluated BCS between weeks 2 – 7 of lactation in 30 cows supplemented with rumen-protected conjugated linoleic acid (**CLA**) and did not find statistical differences, although, this experiment differs to our study in that CLA was the only FA supplemented.

After 30 d in grazing, the difference in BCS observed at 80 DIM was lost because ORG decreased their BCS. Despite this decrement, no differences were observed between ORG and CON cows (ORG: 3.03 ± 0.03 vs. CON: 3.97 ± 0.03; $P$ = 0.17). Body condition score at 50 d after grazing did not differ between ORG and CON cows ($P$ = 0.33). In a similar way, the last BCS evaluation at 150 DIM did not show differences between ORG and CON groups (2.93 ± 0.03 vs. 2.96 ± 0.03 BCS points; $P$ = 0.34 [Figure 8]) and both groups recovered some body condition.

**Table 5.** Overall and stratified by parity (Multiparous and Primiparous) body condition score (BCS) LSM at 1, 3, 7, 21, 80, 110, 130 and 150 DIM of dairy cows supplemented with an organic rumen-protected fat (ORG) con control diet (CON)

| | Overall | | | | Multiparous | | | | Primiparous | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Days in milk | ORG | CON | Difference | *P*-value | ORG | CON | Difference | *P*-value | ORG | CON | Difference | *P*-value |
| 1 | 3.06 | 3.07 | -0.01 | 0.79 | 3.05 | 3.18 | -0.13 | 0.01 | 3.07 | 2.97 | 0.10 | 0.2 |
| 3 | 2.98 | 2.91 | 0.07 | 0.15 | 2.99 | 2.98 | 0.01 | 0.89 | 2.98 | 2.85 | 0.13 | 0.11 |
| 7 | 2.91 | 2.80 | 0.11 | 0.02 | 2.92 | 2.93 | -0.01 | 0.86 | 2.90 | 2.68 | 0.23 | 0.006 |
| 21 | 2.86 | 2.75 | 0.11 | 0.03 | 2.89 | 2.88 | 0.003 | 0.94 | 2.83 | 2.62 | 0.21 | 0.01 |
| 80[a] | 3.08 | 2.87 | 0.22 | <0.0001 | 2.97 | 2.87 | 0.10 | 0.04 | 3.20 | 2.86 | 0.33 | <0.0001 |
| 110[b] | 3.05 | 2.97 | 0.08 | 0.12 | 3.00 | 2.97 | 0.03 | 0.55 | 3.09 | 2.96 | 0.13 | 0.13 |
| 130[b] | 2.79 | 2.84 | -0.05 | 0.37 | 2.71 | 2.87 | -0.16 | 0.003 | 2.88 | 2.82 | 0.06 | 0.46 |
| 150[b] | 2.93 | 2.97 | -0.04 | 0.49 | 2.89 | 2.93 | -0.04 | 0.45 | 2.97 | 3.00 | -0.03 | 0.73 |

[a]: BCS assessment 7 d before grazing; [b]: BCS evaluated during grazing season.

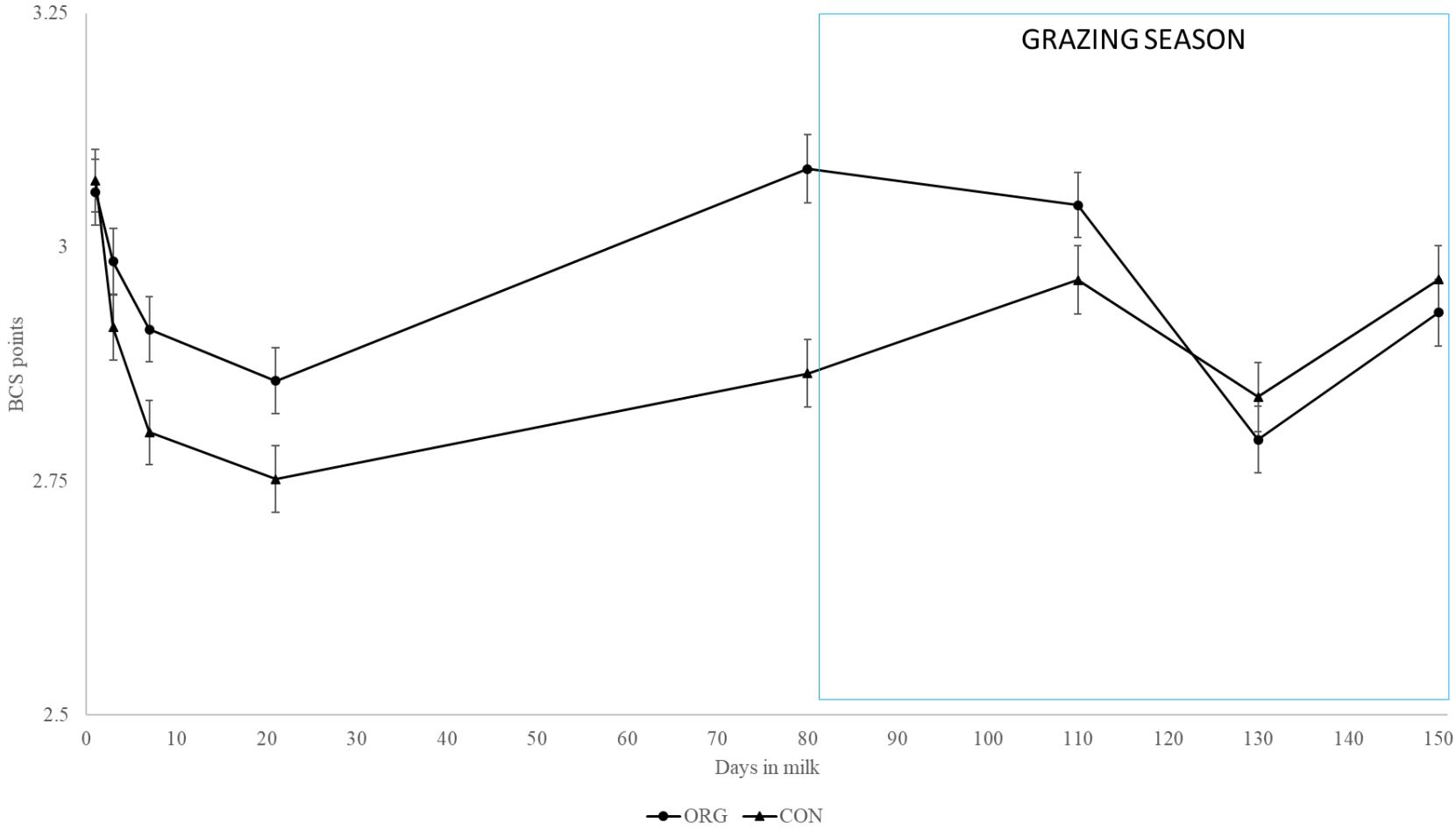


**Figure 8.** Body condition score (BCS) fluctuations by treatment groups (ORG: cows supplemented with rumen protected fat and CON: control cows) during the study period measured at 1, 3, 7, 21, 80, 110, 130 and 150 days in milk. Blue square embraces grazing season started after 80 days in milk. Vertical lines show standard errors.

The interaction between treatment effect and parity on BCS was investigated per evaluation times. BCS LSM, differences and *P*-values of the mean contrasts can be found in Table 5. A significant treatment effect was observed among MP cows at 80 DIM, where MP ORG cows had greater BCS compared to MP CON cows (2.97 ± 0.04 vs. 2.87 ± 0.04; $P = 0.04$). Nonetheless, after 50 d in grazing, MP ORG cows had an abrupt BCS drop and MP CON cows had greater BCS compared to ORG MP cows (2.87 ± 0.03 vs. 2.71 ± 0.04; $P = 0.003$; Figure 9). Similarly to the differences observed between ORG and CON in MP, there were also differences between ORG and CON in primiparous (PP) cows before the grazing season (Figure 9). However, as occurred in MP cows, these differences were not reflected in milk yield since PP cows from both treatment groups produced same milk weights during the trial. This may be explained because PP dairy animals have differential nutrient requirements for growth, maintenance and lactation (Akins, 2016; Heinrichs et al., 2017), which may be subject to specific nutritional management in controlled trials. Conversely to MP cows, there was no difference in the BCS measured during the grazing season. Although, the data from PP cows was characterized for major variability in the BCS reflected by greater variance and standard errors (Figure 9).

Mortality and culling rates were evaluated by treatment groups. Nineteen cows left the study, seven from the ORG group (3.5%) and twelve from the CON group (5.94%; $P = 0.43$). Seven cows died due to respiratory and digestive diseases, one cow from ORG group (0.5%) and six from CON group (2.97%). A tendency was found associating treatment group and the likelihood of death during the study ($P = 0.09$). In the same way, a tendency was found between the survival functions of the treatment groups ($P = 0.06$). Therefore, the hazard of death was 6 times greater for the CON group.

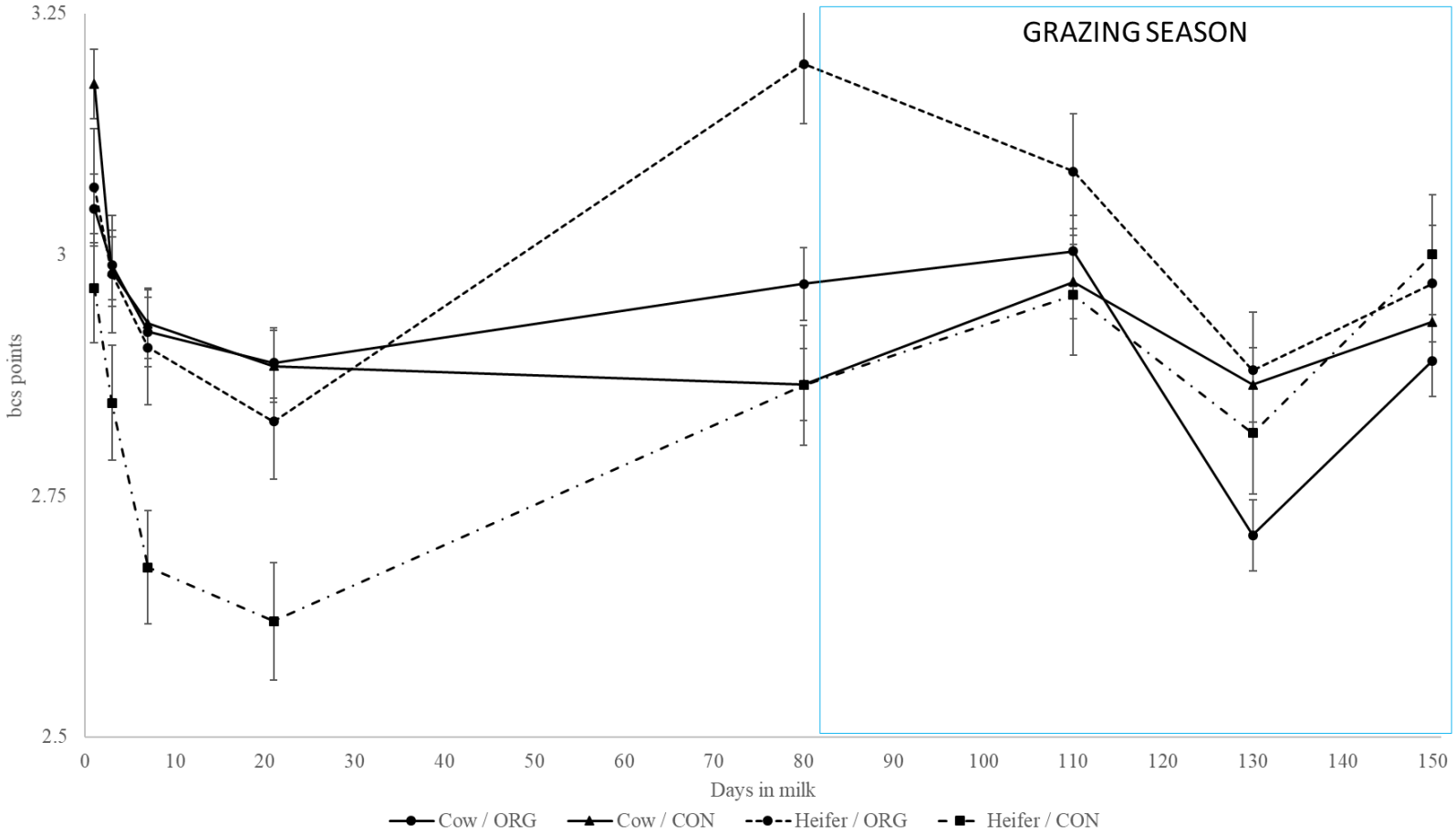


**Figure 9.** Body condition score (**BCS**) fluctuations by treatment group (ORG: cows supplemented with organic rumen protected fat and CON: control cows) and parity (Multiparous: Cow; Primiparous: heifer) during the study period measured at 1, 3, 7, 21, 80, 110, 130 and 150 days in milk. Blue square contains grazing season started after 80 days in milk. Vertical lines show standard errors.

The likelihood of culling due to health reasons such as metabolic, reproductive, locomotion and respiratory diseases did not differ between treatment groups ($P$ = 0.9). Accordingly, no differences were found in the survival functions from both ORG and CON groups ($P$ = 0.9).

*Eating time measurement*

Fat supplementation is recognized to affect DMI (Drackley, 1999; Van Knegsel et al., 2005). When RPF is supplemented to transition dairy cows, DMI has been found to slightly decrease (Hammon et al., 2008; Duske et al., 2009; Lohrenz et al., 2010). In our study, we were unable to daily assess the TMR consumption by treatment groups as research subjects were within the same pen, separated only once a day to receive the treatment pellets. Moreover, separating unconsumed TMR per group was unfeasible due to interference with the normal operation of this commercial dairy farm.

The differences observed after the supplementation of the organic RPF evaluated in this study, at the dose of 0.45 Kg/d, in milk yield and BCS can be attributed to the treatment only if extraneous variables that may confound the associations between the treatment effect and the evaluated outcomes are controlled. In this sense, one of the main variables that could bias these results is DMI of the treatment diets by the experimental units. In the idea of measuring and controlling for DMI, we measured eating time using an ear-tag accelerometer sensor during the pellet supplementation and the rest of the day throughout the study. These devices are becoming more common in the US, and research studies have validated their use to accurately estimate rumination, eating time and activity showing concordance correlation coefficients between 0.7 – 0.99 when contrasted with visual assessment (Borchers et al., 2016; Pereira et al., 2018). Therefore, these devices could represent an opportunity when traditional DMI measurement is not feasible.

According to the overall eating time model within the hour of treatment pellets delivery, there were no differences in eating time by parity ($P = 0.85$) and in the interaction term between treatment group and parity ($P = 0.17$), therefore, these terms were removed from the model. Thus, the final model considered treatment effect only, where eating time did not differ between ORG and CON cows (18.03 ± 0.4 vs. 16.96 ± 0.4 min/h; $P = 0.06$; Figure 10). However, these overall means need a deeper analysis because the number of animals may have differed day by day due to movements to the hospital group and different DIM at the same date. For this reason, a more restricted analysis is needed to state differences among lactation stages. A model considering DIM and the interaction term between treatment group and DIM explored daily differences in eating (min/h) when the treatment pellets were fed (Figure 11). In this model, eating time at pellet delivery differed between treatment groups (ORG: 17.58 ± 0.41 vs. CON: 16.1 ± 0.42 min/h; $P = 0.01$), DIM was significant ($P < 0.0001$) and there was no interaction between treatment groups and DIM ($P = 0.24$).

Although there was a treatment effect on eating time, there are no clear deviations when eating time is assessed continuously through the lactation. Additionally, this data presented wide standard errors that make difficult to state real differences at specific lactation stages. Moreover, in the idea of showing the central tendency of the eating time that can be easily interpreted, weekly eating time during the pellet supplementation was calculated by treatment group. Using this approach, eating time was similar for ORG and CON cows when respective weeks are compared (Table 6).

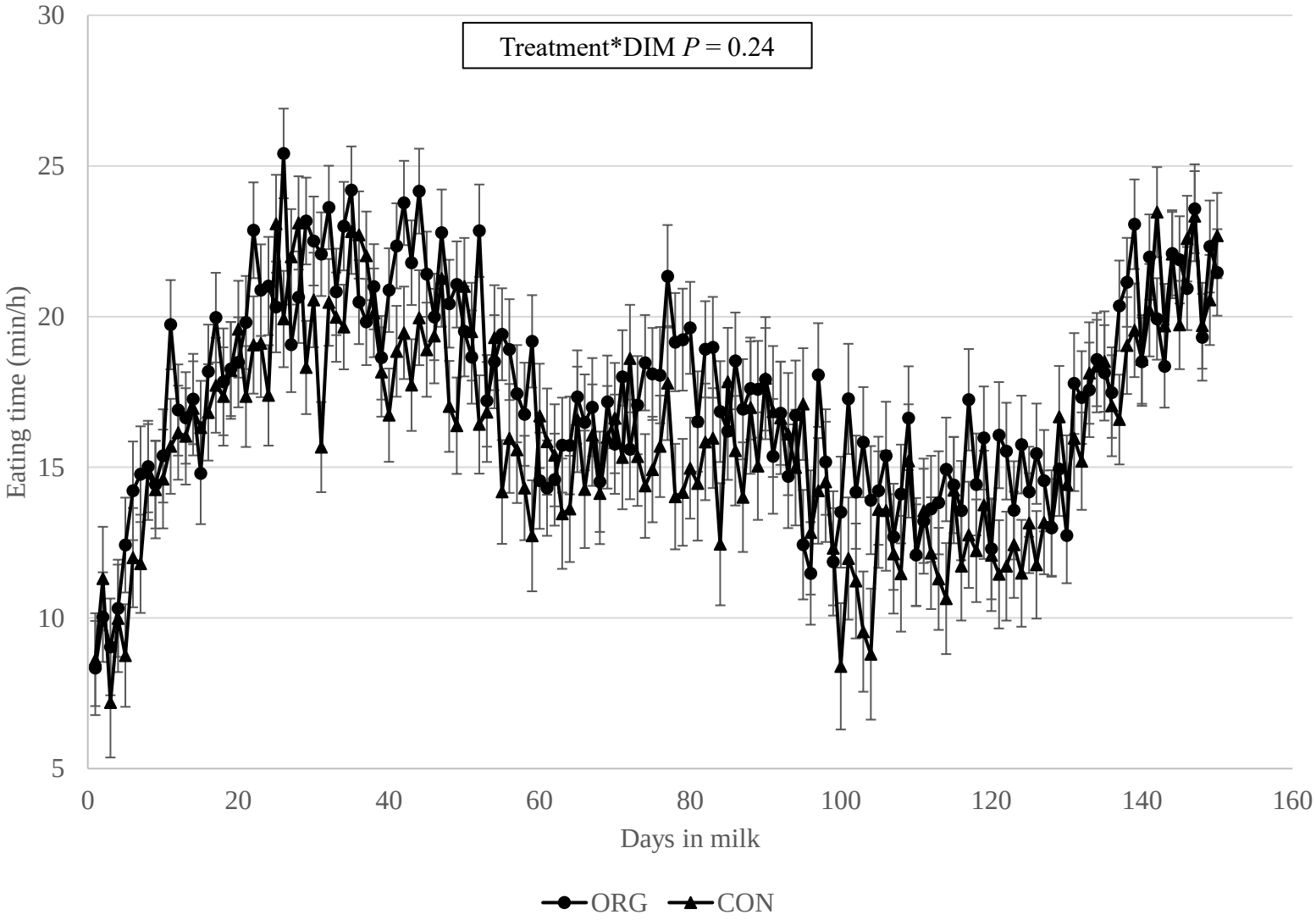


**Figure 10.** Eating time (min/h) restricted to the feeding period of the treatment pellets (between 0700 to 0800 h) of cows supplemented with organic rumen-protected fat (ORG) and control cows (CON).

Overall daily eating time was compared between study groups to investigate possible DMI compensation during the rest of the day that may confound the findings of this study. Throughout the trial, overall eating time did not differ by parity ($P$ = 0.53) and the interaction term $P$-value was 0.11 so that it was retained in the final model. Thus, the eating time of ORG cows was greater compared to CON cows (19.3 ± 0.49 vs. 17.5 ± 0.5 min/h; $P$ < 0.01). The model including the effect of DIM resulted in significant differences between treatment groups (ORG: 19.1 ± 0.4 vs. 17.9 ± 0.4 min/h; $P$ = 0.04), and DIM ($P$ <0.0001). However, no interaction was found between treatment group and DIM ($P$ = 0.91). Daily fluctuation of LSM day eating time (min/h) are showed in Figure 11. In a similar way to the weekly analysis of eating time at the pellet feeding, there was no differences within the respective weeks on the day eating time between treatment groups (table 7). Considering this information eating time tended to increase in a daily basis on cows fed with the organic RPF but there are not differences between ORG and CON cows when eating time is accumulated weekly, either at treatment pellet delivery or at the rest of the feeding during the day.

**Conclusions**

Under this study settings, the study results indicate that supplementation of 0.45 kg/d/head of organic rumen-protected fat increased daily and total milk yield up to 150 DIM and improved body condition score after calving up to 80 DIM. The inclusion of the tested supplement did not cause differences or detrimental effects on milk fat and protein, serum glucose, BHB and NEFA, reproductive performance, and eating time. Finally, cows fed with the organic rumen-protected fat tended to have lower culling likelihood up to 150 DIM. Overall, the productive and energy balance indicators were similar to studies on rumen-protected fats tested in conventional dairy herds. Thus, the evidence presented in this study suggests that the energy density granted by the organic rumen-protected fat was devoted for milk production and maintenance of body condition

and could be used in organic herds for improvement of such responses. Future research should address the understanding of the effects of energy source on specific metabolic and immune pathways affected by the rumen-protected fats, as well as which FA profiles provide better productive and health performance in transition dairy cows.

**Table 6.** Eating time during the daily supplementation of the treatment pellets (0700 to 0800 h) during the trial (1 to 150 DIM)

| Eating time (min/h) | | | | | |
|---|---|---|---|---|---|
| Weeks of study | ORG | SE | CON | SE | *P*-value |
| 1 | 11.90 | 0.66 | 10.85 | 0.69 | 0.99 |
| 2 | 16.49 | 0.68 | 15.53 | 0.71 | 0.99 |
| 3 | 18.32 | 0.70 | 17.61 | 0.71 | 0.99 |
| 4 | 21.50 | 0.69 | 20.63 | 0.72 | 0.99 |
| 5 | 22.77 | 0.66 | 19.72 | 0.67 | 0.35 |
| 6 | 20.99 | 0.65 | 19.79 | 0.68 | 0.99 |
| 7 | 21.68 | 0.65 | 18.62 | 0.70 | 0.4 |
| 8 | 19.31 | 0.69 | 17.70 | 0.74 | 0.99 |
| 9 | 16.08 | 0.71 | 14.92 | 0.77 | 0.99 |
| 10 | 16.33 | 0.71 | 15.34 | 0.76 | 0.99 |
| 11 | 18.07 | 0.71 | 15.93 | 0.76 | 0.99 |
| 12 | 18.51 | 0.72 | 14.63 | 0.78 | 0.12 |
| 13 | 17.23 | 0.73 | 16.20 | 0.82 | 0.99 |
| 14 | 15.07 | 0.76 | 15.25 | 0.82 | 0.99 |
| 15 | 14.39 | 0.78 | 10.97 | 0.84 | 0.59 |
| 16 | 13.96 | 0.75 | 12.90 | 0.80 | 0.99 |
| 17 | 14.90 | 0.74 | 12.38 | 0.77 | 0.96 |
| 18 | 14.68 | 0.72 | 12.04 | 0.77 | 0.91 |
| 19 | 15.43 | 0.71 | 15.26 | 0.74 | 0.99 |
| 20 | 19.65 | 0.68 | 18.22 | 0.70 | 0.99 |
| 21 | 21.18 | 0.65 | 21.55 | 0.67 | 0.99 |
| 22 | 19.76 | 0.68 | 19.84 | 0.68 | 0.99 |

ORG[a]: Cows supplemented with organic rumen-protected fat; CON[b]: cows supplemented with control pellets; *P*-value[c]: LSM comparison between treatment groups by weeks of study.

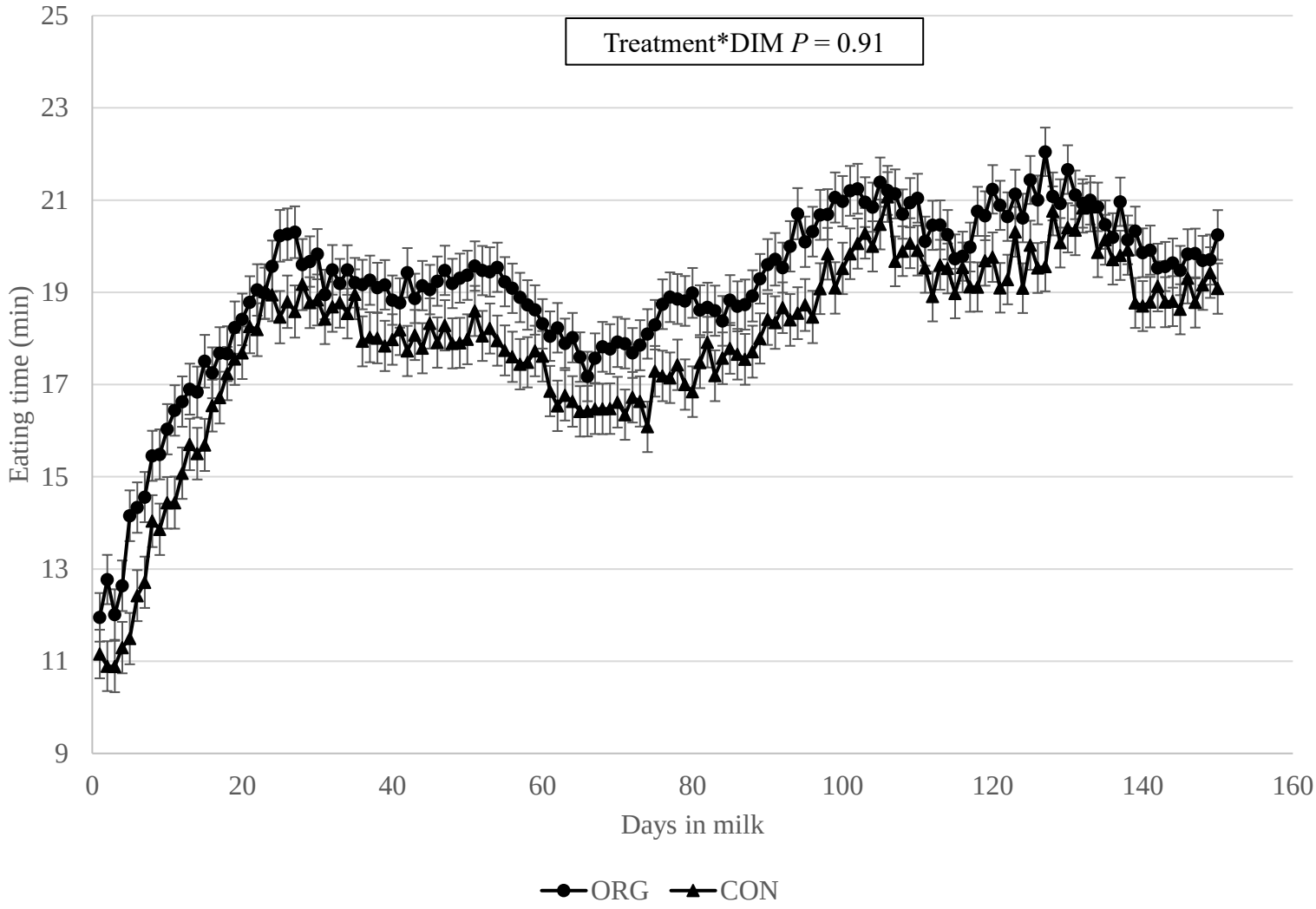


**Figure 11.** Daily eating time (min/h) restricted to the feeding period of the treatment pellets of cows supplemented with organic rumen-protected fat (ORG) and control cows (CON).

**Table 7.** Overall eating time by weeks during the supplementation of the treatment pellets and during the rest of the feeding times.

| Eating time (min/h) | | | | | |
|---|---|---|---|---|---|
| Weeks of study | ORG | se | CON | se | *P*-value |
| 1 | 13.36 | 0.41 | 11.95 | 0.41 | 0.94 |
| 2 | 16.24 | 0.42 | 14.72 | 0.42 | 0.89 |
| 3 | 17.93 | 0.42 | 17.07 | 0.42 | 0.99 |
| 4 | 19.70 | 0.42 | 18.73 | 0.42 | 0.99 |
| 5 | 19.39 | 0.41 | 18.71 | 0.42 | 0.99 |
| 6 | 19.10 | 0.41 | 17.95 | 0.42 | 0.99 |
| 7 | 19.18 | 0.41 | 18.02 | 0.42 | 0.97 |
| 8 | 19.38 | 0.41 | 18.02 | 0.42 | 0.99 |
| 9 | 18.38 | 0.41 | 17.20 | 0.42 | 0.99 |
| 10 | 17.69 | 0.41 | 16.50 | 0.42 | 0.94 |
| 11 | 18.21 | 0.41 | 16.76 | 0.42 | 0.97 |
| 12 | 18.70 | 0.42 | 17.34 | 0.42 | 0.99 |
| 13 | 19.09 | 0.42 | 17.91 | 0.42 | 0.93 |
| 14 | 20.28 | 0.42 | 18.82 | 0.42 | 0.99 |
| 15 | 21.09 | 0.42 | 19.91 | 0.42 | 0.99 |
| 16 | 20.79 | 0.41 | 19.86 | 0.42 | 0.99 |
| 17 | 20.22 | 0.41 | 19.36 | 0.42 | 0.95 |
| 18 | 20.99 | 0.41 | 19.58 | 0.42 | 0.99 |
| 19 | 21.24 | 0.41 | 20.40 | 0.41 | 0.99 |
| 20 | 20.40 | 0.41 | 19.56 | 0.42 | 0.99 |
| 21 | 19.68 | 0.41 | 18.88 | 0.42 | 0.99 |
| 22 | 19.82 | 0.42 | 18.99 | 0.42 | 0.9 |

ORG[a]: Cows supplemented with organic rumen-protected fat; CON[b]: cows supplemented with control pellets; *P*-value[c]: LSM comparison between treatment groups by weeks of study.

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# CHAPTER 2 - CASE REPORT: ASSESSMENT OF HUMAN-CONDITIONED SORTING BEHAVIOR OF DAIRY COWS AND ITS USE IN FARM RESEARCH TRIALS

## Introduction

The improvements in several fields of the dairy sciences reached through controlled studies must be available to dairy farmers so that new technologies and methods progress animal health, profitability, food security and sustainability. Thus, applied science within a productive context represent a valuable tool to implement new strategies based on published evidence. Nonetheless, productive settings bring new challenges to test specific effects to new products and/or managements practices. Moreover, other factors such as labor, climatic conditions, milking schedules, lack of technical resources and unpredictable events make it more challenging to conduct on-farm experiments. Therefore, a systematic approach in the evaluation of interventions on determined outcomes should be considered in such trials, together with a careful consideration that can bias the conclusions made. The study presented in chapter I, evaluated the effect of an organic rumen protected fat (**RPF**) on performance and health of dairy cows. As discussed in chapter II, the accurate delivery of the treatment diets and the estimation of dry matter intake (**DMI**) are key for the validity of the study. One of the main problems we had to overcome for individually feeding of the study cows was the group separation. This was because we had only one pen available that had to be divided every time the treatment pellets were fed. These efforts were made to consider cow as experimental unit (N = 202) rather than the pen (N = 2). As there was no technical feasibility to automatically separate the study cows, manual sorting performed by farm operators was used as separation method. This method relies in the evidence that suggest that dairy cattle behavior can be modeled to facilitate farm management procedures.

Research has shown that dairy animals are able to acquire behavioral responses through learning processes, lead either by their herd mates or by farm management (Costa et al., 2014; De Paula Viera et al., 2012). For example, the use of the Calan gate systems in nutritional studies is accepted as an individual feeding method. However, this requires a training period, usually of three weeks, after which cows can eat properly from their individual feeding bins (Holcomb et al., 2001; Yang et al., 2017). The learning abilities exhibited by dairy cattle are formed by their complex social structure, where animals learn through behavioral synchronization to novel elements (DeVries et al., 2004) and to actions by their neighbors that produce a conditioned behavior (Duve et al., 2012; Mainardes and DeVries, 2016). Dairy animals learn from individual trial and error, however, there is an important component of group learning, especially in young animals from older individuals (Duve et al., 2012). In this sense, the social structure might help to show desired behaviors in the productive systems. Conversely, socially hierarchy could cause negative social interactions, such as competition for feeding space that may result in increased stress in subordinated animals, affecting their eating, and resting time, as well as their productive performance (Grant et al., 2001; DeVries et al., 2004; Crossley et al., 2017). Another important component of cow behavior is the interaction with the environment, which is shaped by the cow-facilities and the cow-human interactions. Research studies have shown that the design of housing affects feeding and resting behaviors, as well as other health and productive responses on lactating dairy cows (DeVries et al 2004; Cook et al., 2009; Kull et al., 2017). On the other hand, cow-human interaction has important implications for animal welfare and productivity (Waiblinger et al., 2006; Lürzel et al., 2016), with lasting effects in the learning process of dairy cows. A possible explanation is that dairy cows' perception of human activities models their behavior, based on the emotions produced by such activities (Waiblinger et al., 2006), which can become conditioning

factors to behaviors such as group movements, fear signs, or isolation and separation of a group of animals.

*Study hypothesis and general objective*

Cattle movement and sorting performed by humans is one of the main tasks in a dairy farm. Such activities result in cows becoming accustomed to human handling, associating human vocalization and/or body language to specific behaviors or reactions (Lürzel et al., 2016). Therefore, a better understanding of the impact of the human component as a conditioning factor on the behavior of lactating dairy cows could be of assistance in farm labor and in on-farm research studies requiring individual animal or group sorting. In this study, we hypothesized that, after a training period, dairy cows become conditioned to human sorting, allowing for correct separation in two subgroups. Consequently, the objective of this study was to evaluate the effectiveness of three different sorting methods on the placement of lactating dairy cows in separated pens.

**Materials and Methods**

*Animals and housing*

The animals used for the assessment of the sorting behavior were part of the study described in chapter II. This study was conducted from July 6 to July 16, 2017. One-hundred and seventy-six Holstein cows (49 primiparous [**PP]** and 127 multiparous [**MP;** parity = 2.5 ± 1.3; mean ± SD]) in a commercial dairy farm located in Northern Colorado were evaluated. The study cows were part of a parallel nutritional trial on RPF supplementation, where individuals were sorted daily into two contiguous sub-pens. For the behavioral study, cows were assigned into two treatment groups, each blocked by parity (A= 2.6 ± 1.3; B = 2.4 ± 1.3 lactations). The study animals were housed in the same pen provided with 200 free stalls with sand bedding. The research pen included a barn (156 m x 11.3 m; length x width) and access to outdoor space (156 m x 36 m,

Figure 12). All cows in the study had ad libitum access to drinking water from 4 automatic drinkers. The milking schedule was 3 times per day (0700, 1500 and 2300 h) in a rotatory milking parlor of 50 stalls.

*Experimental design*

In the nutritional trial, the animals were randomly assigned to one of two treatments 15 days before of the expected due date. Treatment group A included 91 animals (25 PP and 66 MP) supplemented daily with 1.5 Kg of an experimental pellet, formulated to contain 28% of an organic rumen protected fat (Organilac, Organic Animal Nutrition, Boulder, CO. Pellets were elaborated by Ranch-Way Feeds, Fort Collins, CO). Group B consisted of 87 animals (24 PP 61 MP) fed with a control pellet. At enrollment, all study animals were affixed with an ear tag consisting of an accelerometer measuring general activity, eating time, and rumination (Cow-Manager, distributed by Select Sires Mid-America, Logan, UT).

Within the day of calving, animals were moved into study pen to start the sorting training and the feeding of the treatment pellets from 1 to 150 days in milk (**DIM**). Both groups were fed with the same total mixed ration (**TMR**) and the treatment pellets were served only after the morning milking (0700 h) on top of the TMR.

*Sorting procedures and data collection*

To feed the treatment pellets, one farm operator individually sorted the animals at the entrance gate of the research pen after the morning milking (0700 h). Sorting of the study cows was assisted by plastic color links (A = red; B = green) attached to the ID tags. Additionally, cows in group B had a collar for better identification. A temporary gate was located at the center of the pen to divide the groups within the research pen (Figure 12). After one hour, cows were released from headlocks and the gate dividing both sub pens was opened.

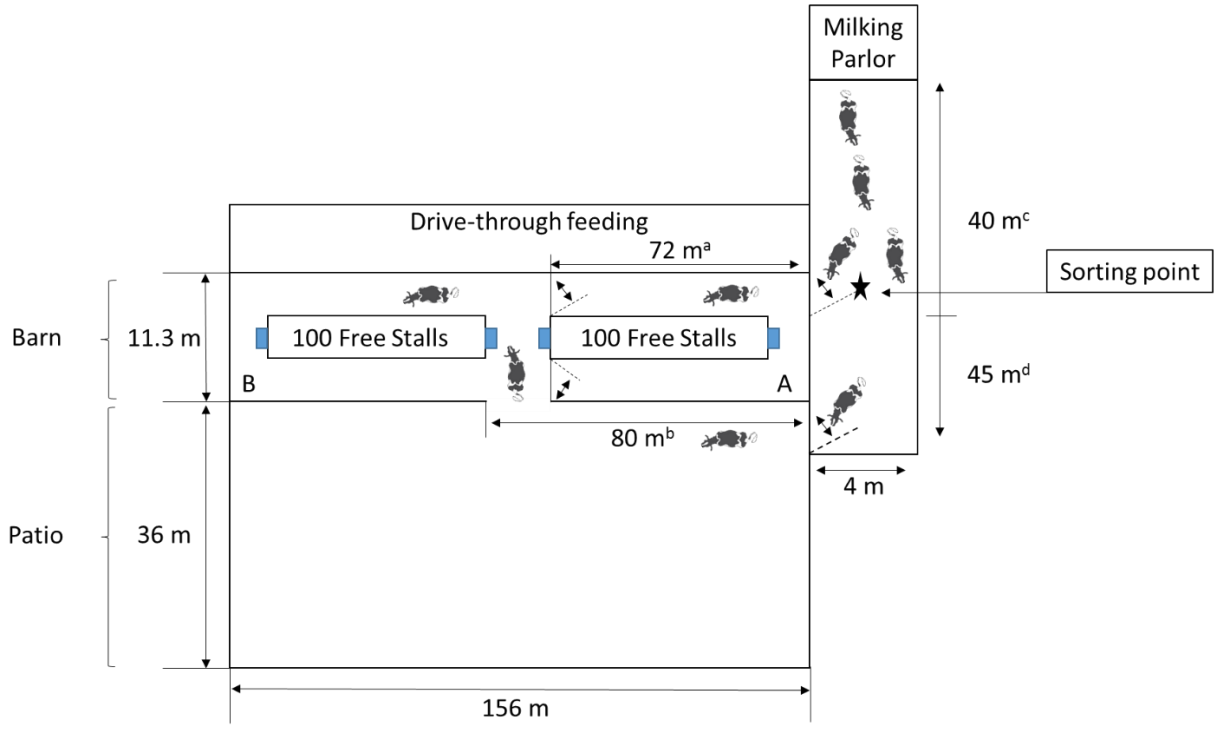


**Figure 12.** Layout of the research pen used for the sorting behavior evaluation. $m^a$ = distance from the entrance gate to the center of the treatment pen; $m^b$ = distance from the entrance gate of the patio to the pen back gate; $m^c$ = distance from milking parlor to the entrance gate of the treatment pen; $m^d$ = distance from milking parlor to the entrance gate of the control pen. $m^a + m^c$ = walking distance of the treatment cows from the exit of the milking parlor to the center of the research pen; $m^b + m^d$ = walking distance of the control cows from the exit of the milking parlor to the center of the research pen. Dashed line = temporary gates).

Starting at day 164 ± 6.3 of the nutritional trial, the correct placement of each cow was recorded in both groups. Three sorting managements were sequentially tested to investigate the conditioning factor of the observed self-sorting behavior. 1) active manual sorting (**AS)** = the animals were led to their correspondening pen by displacements of the gate (Figure 13a). 2) Passive sorting (**PS)** = the person sorting the cows stood on the center of the alley with the door open without any further intervention (Figure 13b). 3) Gate sorting (**GS**) = the gate at the sorting point was open leaving 2 m of alley for each group, with no human interaction. Each cow was observed daily for 15 d, where AS, PS and GS were assessed in a sequence of 5 days. This order was chosen to transition from a greater to a smaller degree of intervention. After sorting, cows from each group had individual access to the treatment pellets and TMR through headlocks to allow researchers to perform a head counting and other procedures related to the nutritional trial.

The same person was at the pen gate during at all observation points. Cows in group A had direct access to the study pen after walking the transit alley, whereas cows in group B rounded the pen to enter using a back gate (Figure 12). For group A, the waking distance to the center of the pen was 132 m, whereas the group B walked 145 m to the same point. After one hour, all cows were released from the head locks and the temporary gate was opened providing free access to all the areas of the pen. All study cows were subject to the same activities and management during the rest of the day, milking schedules, feeding times, and grazing.


**Figure 13.** Methods of animal separation. A) Image of the sorting procedure during the training period. During return from the morning milking (0700 h), Group A animals were directed to the left of the person sorting while the group B was directed to the right. B) Image of the passive sorting. Note the individual and group self-separation after seeing the person standing by the pen gate (cows approaching to the dividing gate).

*Statistical analysis*

Descriptive analyses for lactation number and DIM were performed using the MEANS and FREQ procedures of SAS (SAS 9.4, SAS institute Inc., Cary, NC). The sorting efficacy assessed by the mean of animals allocated correctly during the observation period was compared between sorting treatment using the non-parametric Wilcoxon ranked test (PROC NPAR1WAY). In the same idea of assessing the sorting methods, an error rate (misplaced cows [n/d]) was calculated to compare the overall success of separating cows among the sorting treatments. PROC MIXED of SAS was used including study group, sorting treatment and an interaction term in the model. Additionally, an individual error index was calculated averaging the times that individual cows went to the wrong side of the pen throughout the observation period. To test the effect of group, parity and general activity ratio (average of daily rumination [min]/daily activity [min] during the observation period) on the error index, PROC MIXED of SAS was used. Furthermore, an interaction term between group and parity was included. Statistical significance was determined at $P< 0.05$.

**Results and Discussion**

During the nutritional trial, researchers noted that all cows had acquired the behavior of allocating themselves in their corresponding sub-pen, as soon as they came out of the milking parlor. After seeing the sorter standing on the pen gate, cows formed two separated lines leading to their correct section of the pen (Video 1, in Appendix), where cows seemed confident about their place. Lürzel et al (2016) and Phillips et al (2015) have evaluated the modification of cow behavior due to human presence in heifers and mature cow. Both authors reported modifications of animal behavior in response to human interaction, especially to novel situations. Furthermore, Lürzel et al (2016) have suggested that positive interactions, causing comfortable emotions in dairy

cattle, could be employed to improve cow-human relationships during farm activities, such as animal sorting. Another evidence of dairy animals training relates to the use of operant conditioning of urination on pre-weaned dairy calves (Vaughan et al., 2014), which conditioned their urination habits to specific places and handling. However, there is a lack of research on operant conditioning on lactating cows.

The transit from the milking parlor to the housing pen is a familiar situation for dairy cows, which could be modified and reinforced by human sorting conditioning. In our study, the walking behavior showed by the cows was present both as individual conduct and as group behavior. When cows approached the operator at the pen gate they diverted themselves towards their assigned pen, facilitating cow separation throughout the nutritional trial (Figure 13). Accordingly, our research question was oriented at determining what was the conditioning factor causing the observed behavior; it could be either the human presence at the gate or the gate itself. A secondary question was whether passive sorting could be used for future farm management or research studies after sorting training.

Our hypothesis was that cows would remember their access direction to the pen after seeing the person standing at the entrance gate and that AS would be more effective than PS; while these two methods would be more effective than GS. Supporting this idea, we observed that the AS sorting did not require much effort, as most cows had learned their way during the nutritional trial (Video 1, in Appendix).

The total number of animals correctly placed when AS was applied is presented in Table 8. During AS, few cows seemed eager to eat but their direction was corrected with simple gate movements.

Only one cow was misplaced using AS. When the ranked means of the total of animals correctly placed in the pen were compared, AS and PS methods were significantly different ($P$ = 0.005), with AS showing a greater efficiency in AS. Despite this difference, PS showed to be a comfortable method for the cows and a similar conditioned behavior can be observed (Video 2, in Appendix). Overall, 99.8% of the animals were correctly sorted, as it was also established during the previous nutritional trial (data not shown). The differences on the ranked means might be explained because as AS and PS sorting methods were evaluated subsequently and during different days where the cows seemed to forget their place as PS advanced over time. This situation showed a linear tendency decreasing from 97.7% of correctly placed cows to 92.6% on the last day of PS (Table 8). On the other hand, it could be argued that cows in group B faced a greater challenge when compared to group A cows because they had a more diverted route to their section of the pen and, for this reason, they had greater error rates than those in the treatment group.

The overall average error rate during the observation period per study group was 1.5 ± 4.8 and 8.5 ± 14.3 animals per day for A and B groups, respectively. When the error rate of the study groups was observed by sorting treatment, a significant interaction was found between those effects ($P$ = 0.0003) that might explain the greater error in the group B cows when PS was evaluated.

Non-human gate sorting could not be evaluated accurately, as the absence of the person at the sorting point resulted in all the cows entering directly to the A pen overcrowding the area. For this reason, the temporary gate dividing the research pen had to be opened to ensure access to feed and water as well as to avoid injuries in the cows. This supports the idea that the sorting behavior of the cows was modulated, in a major extent, by the active and the passive sorting. Considering AS and PS, both treatment groups showed an operant conditioning to human sorting because the

behavior of self-separation remained only when the person sorting was present. On the contrary, the absence of the person at the pen gate (GS) provoked random movement of the cows towards the pen, regardless of the treatment group. Although the sorting behavior was not completely induced by GS, the cows seemed confused by the gate opening direction making some cows take the correct direction (Video 3, in Appendix). In this study, cows may have associated the person's correction movements as the operant conditioning factor that reinforced the desired behavior of self-sorting. As cows going to the wrong place were corrected in AS, this may have cause unpleasant emotions such as anxiety or frustration (Waiblinger et al 2006). Those emotions were relieved as soon as cows walked to the correct pen; hence, the unpleasant emotions became pleasant emotions since they were rewarded with a free way to the feeding bunk.

An individual error index was calculated to investigate individual differences between cows that were repeatedly misplaced. The maximum number of times that the same cow went to the wrong side was 5 times (error index = 0.45). Twenty-two cows (8 PP and 14 MP) had an index > 0.1, which means they were wrong more than twice. PP cows have been reported to be more likely to block the way of other cows compared to MP cows (Jacobs et al., 2012). However, in our study there were no differences between parity on the least square means of the error index ($P$ = 0.15) and the interaction between parity and group was not significant. Additionally, we evaluated whether the animals that were misplaced more often had a different pattern of activity, using a rumination by activity ratio as a measure of general activity during the observation period. This parameter did not differ between animals with error index greater than 0.1 compared to those with lower values (197.7 ± 7.7 vs. 195.3 ± 2.8 units; $P$ = 0.7).

**Table 8.** Total number (percentage) of cows correctly allocated in each treatment sub-pen by sorting treatment during the 15 d observation period

| | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | AS[a] | | PS[b] | | GS[c] | | |
| Observation day | Total | Correct (%) | Total | Correct (%) | Total | Correct (%) | *P-value*[d] |
| 1 | 176 | 176 (100) | 176 | 172 (97.7) | 176 | 62.8 | |
| 2 | 174[f] | 173 (99.4) | 176 | 168 (95.5) | 176 | Inac[e] | |
| 3 | 176 | 176 (100) | 175[f] | 165 (94.3) | 176 | Inac[e] | |
| 4 | 176 | 176 (100) | 175[f] | 165 (94.3) | 176 | Inac[e] | |
| 5 | 175[f] | 176 (100) | 176 | 163 (92.6) | 176 | Inac[e] | |
| Mean | | 175.2 | | 166.6 | | - | 0.004 |
| Standard deviation | | 1.3 | | 3.5 | | - | |

[a] AS: active sorting; [b] PS: passive sorting; [c] GS: gate sorting.
[d] *P*-value calculated using the Wilcoxon non-parametric test for the comparison between AS and PS ranked means. GS was not included in the analysis because only one observation was recorded accurately.
[e] Inac: Inaccurate head counting on both treatment pens due to overcrowded side of one pen for which temporary gate were opened because of welfare assurance.
[f] The number of total cows differs because movements to the hospital group.

**Table 9**. Total number (percentage) of cows correctly allocated in each treatment pen by sorting treatment and by study group

| Observation day | Sorting treatment | | | | | |
|---|---|---|---|---|---|---|
| | AS | | PS | | GS | |
| | Treatment | Control | Treatment | Control | Treatment | Control |
| 1 | 91 (100) | 85 (100) | 91 (100) | 81 (95.3) | 74 (82.2) | 36 (42.3) |
| 2 | 90 (98.9) | 82 (100) | 91 (100) | 77 (90.6) | Inac[a] | Inac[a] |
| 3 | 91 (100) | 85 (100) | 90 (100) | 75 (88.2) | Inac[a] | Inac[a] |
| 4 | 91 (100) | 85 (100) | 91 (100) | 74 (88.1) | Inac[a] | Inac[a] |
| 5 | 90 (100) | 85 (100) | 91 (100) | 72 (84.7) | Inac[a] | Inac[a] |

[a]Inac: Inaccurate head counting on both treatment pens due to overcrowded side of one pen for which temporary gate were opened because of welfare assurance.

As with the proportions of cows correctly placed, the individual error index was higher for the B group compared to the A group (0.09 ± 0.01 vs. 0.02 ± 0.01; $P < 0.0001$). We investigated general activity, rumination and parity because these have been identified as factors affecting the social behavior of dairy cows (Maekawa et al., 2002; Neave et al., 2017). Nonetheless, none of these parameters were associated with the sorting behavior, when we compared cows with higher error index to cows with lower error indexes.

The reactions to novel events varies between animals (Van Reenen et al., 2004) and the presence of the gates or alleys can affect cow traffic and walking behavior (Jacobs et al., 2012). The reason why the same animals were misplaced at all time points when the PS was applied remains unclear. Nonetheless, this may be due to inherent cow reactions related to temperament, which may have a genetic component (Haskell et al., 2014). Besides genetic factors, environmental stimulus may play an important role in the ability to learn a new behavior. External variables, such as the waiting period on the milking parlor affect cow behavior, especially in the eagerness to eat and drink. Additionally, the variation in the waiting period can cause disruptive social relationships in the group, making some cows to be less prone to be trained on operant conditioning (Dijkstra et al., 2012). Despite all those factors, the cows observed in this study showed a clear group behavior that was very useful in the success of the parallel nutritional supplementation trial because it was comfortable for the cows and for the operators.

Taking into consideration the results of this study, the research line for studying cow sorting behavior should include the time that cows take to learn the self-sorting behavior, how long does it take for such behavior to be lost and whether previously trained cows are able to resume the acquired behavior when the operant conditioning factor is reapplied.

## Conclusions

Cows evaluated in this study showed a conditioned behavior of self-sorting as response to human sorting regardless of the sorting methods utilized. The sorting behavior disappeared when the person was not present. Active sorting had the greatest efficiency on sorting dairy cows in two subsets of a lactating pen. Passive sorting had also high performance for cow separation, but it favored greater individual error rates. Individual errors rates were not associated with parity and general activity. After training, after a period of training, lactating dairy cows became operant conditioned for human sorting, which represents an opportunity to perform animal separation without additional costs or animal discomfort.

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# CHAPTER 3 - RELATIONSHIP BETWEEN FLUCTUATIONS OF BEHAVIORAL PARAMETERS AND CULLING AND PRODUCTION LEVEL IN PERIPARTURIENT DAIRY COWS

## Introduction

The development and the growing use of remote sensor devices (**RSD)**, monitoring dairy animals for several behavioral and physiological variables, such as rumination and eating, activity, locomotion, lying behavior, and body temperature, in commercial dairy farms creates new opportunities for research on the associations between behavior and the onset of disease signs, dynamics of dry matter intake, welfare assessment, calving behavior, and resulting effects on productivity (Friggens et al., 2007; Liboreiro et al., 2015; Beauchemin, 2018). In the last decade, studies have recognized the value of RSD for disease prediction, as well as for behavioral monitoring related to reproductive performance and impaired health (Alsaaod et al., 2015; Liboreiro et al. 2015).

Most of the technology behind RSD relies on tridimensional accelerometers and pressure sensors that associate animal's movements with activities such as rumination, eating, non-active and active time, steps, lying bouts, and resting time. The location in the animal and the interface of data recording of the RSD variate according to the manufacturer. For example, ear-tag devices (e.g. CowManager SensOor, Agis Automatisering BV, Harmelen, the Netherlands; Smartbow GmbH, Jutogasse, Austria) have been validated for monitoring rumination, eating, and drinking in dairy cattle by concordance correlations coefficients with visual observation (Pereira et al., 2018; Roland et al., 2018).

On the other hand, other devices have combined accelerometers and microphones in collars for monitoring of activity and rumination (HRLD collars, SCR Engineers Ltd., Netanya, Israel). These data have been used to characterize activity and rumination behavior in cows affected by metabolic disorders and metritis (Liboreiro et al., 2015; Paudyal et al., 2016). Additionally, pedometers have been designed using tridimensional accelerometer technology (AfiAct Pedometer Plus, Afimilk, Kibbutz Afikim, Israel; HOBO Pedant G, Onset Computer Corp. Pocasset, MA; CowAlert IceQube, IceRobotics Ltd. Edinburgh, Scotland; Track A cow, ENGS, Rosh Pina, Israel) not only for monitoring locomotion behavior and claw lesions but also for monitoring eating behavior (Borchers et al., 2016; Nechanitzky et al., 2016; Roland et al., 2018).

The adoption of these technologies by dairy farmers and researchers requires sound evidence about the accuracy and precision of the measures provided by RSD, as this information could reduce labor costs and improve profitability. Recent studies have assessed the reliability of the RSD data contrasting their readings with visual evaluations of the behavioral variables using concordance correlation analysis (Borchers et al., 2016; Pereira et al., 2018). Borchers et al. (2016) found correlations ranging between 0.7 and 0.99 when observations were contrasted to rumination, eating time, activity and lying behavior data in lactating cows. They concluded that RSD accurately monitor dairy cattle behavior. In agreement with those results, Pereira et al. (2018) concluded that the ear-tag sensor (Cow Manager ®) accurately measured rumination and eating time in grazing dairy cattle, reaching correlations between 0.71 and 0.88. Due to this evidence, dairy farmers interest into acquiring these technologies has risen. In this sense, a survey performed by Garguilo et al. (2018) determined that the adoption of RSD technology would increase significantly by 2025, but they also recognized that their use would depend of herd size since larger dairies are more likely to adopt precision dairy technologies.

Since 1980, sensors for measuring parameters from individual cows started being developed (Rutten et al., 2013). Nonetheless, over the last ten years researchers have started to build a body of knowledge about the use of RSD in dairy sciences, as well as data analysis approaches. Sensor devices represent an opportunity for continuously monitoring dairy cattle behavior and contribute to the understanding of subclinical disease at individual level, as well as in population medicine. Liboreiro et al. (2015) determined that postpartum dairy cows affected with subclinical ketosis have differential patterns of rumination during the first 21 DIM. Additionally, exploratory studies using RSD have recognized the potential that behavioral monitoring could improve calving prediction using cow activity, rumination, and ear temperature (Rutten et al., 2017). In agreement with this idea, previous studies performed by our group have found correlation patterns between behavioral variables around time of clinical diagnosis depending on specific diseases, suggesting potential as discriminatory tool of specific health disorders (Paudyal et al., 2016). However, more research is needed to determine which behavioral and physiological variables, monitored by RSD, better reflect specific health disorders (Rutten et al., 2013; Liboreiro et al., 2015).

Despite the increase in the use of RSD by large dairy farms, there is a lack of research on the use of RSD in forecasting models for disease detection or classification of health status. A possible explanation for this research gap is the complexity of the information, which require intense data mining, it is highly autocorrelated, and does not always meet the assumptions of statistic analysis such as independency and/or normal distribution (Shumway and Stoffer, 2016). As RSD data is continuously recorded in adjacent sampling points, usually by minutes or seconds, the study of time-related changes is an important factor that should be accounted in the data analysis (Friggens et al., 2007).

Few studies evaluating fluctuations of behavioral variables and metabolites in healthy and sick animals, as well as some validation studies, have analyzed RSD data through simple correlations (Liboreiro et al., 2015; Borchers et al., 2016; Pereira et al., 2018), which neither consider the time component nor the autocorrelation of the data originated from the same animals at adjacent sampling points. Additionally, in previous studies by our group, we have noted that fluctuation of behavioral variables around the time of diagnosis and calving have notorious trends that should also be considered in the analysis (Paudyal et al., 2016). Moreover, we have observed that fluctuations of behavioral variables are time-related in specific diseases or health status, showing some reciprocity either leading or lagging fluctuations of other variables over time. Observations collected sequentially in time are defined as time series data (Milhøj, 2013); therefore, measurements collected by RSD meet this assumption and can be considered as non-independent data (de Mol et al., 1999). For all these distinctive characteristics, the time series analysis (**TSA**) seems a reasonable approach to investigate future applications of RSD data in predictive models of disease in peripartum dairy cows.

Some studies have previously used TSA for continuous data collected from individual dairy cows. Deluyker et al. (1990) proposed autoregressive stochastic models for short-term forecasting of daily milk yield that could be used in automated milking systems. Additionally, they suggested that changes in milk yield and DMI preceded mastitis diagnosis, and that such variables could be cross-correlated overtime. In agreement, temporal relationships between two time moving variables have been described previously in dairy cows. Procknor et al. (1986) described the relationship of the pulsatile fluctuation of luteinizing hormone (**LH**) and progesterone using cross-correlation analysis (**CCA**).

This study was useful to determine that LH peaks lead to a progesterone peak in a lag of 10 minutes. Thereby, extrapolating these analyses OF the temporal relationships between behavioral variables could be useful to investigate differential deviations in behavioral data by health status, as well as to identify time lags in behavioral variables that might be predictors of others in a context of disease or production.

Recent studies have explored the application of TSA. Friggens et al. (2007) tested a time series model for the risk of mastitis based on serial measurements of milk lactate dehydrogenase and milk yield. The authors compared model effectivity to detect mastitis versus the traditional cut-off established by measuring somatic cells, determining that these models are as accurate as other mastitis detection systems. Additionally, Friggens et al. (2007) recognized that binary classification models are not appropriate to study time related changes. Finally, the researchers concluded that models accounting time fluctuation of the studied variables could differentiate mastitic and healthy cows 4 d before diagnosis and treatment on the farm.

*Study hypothesis and general objective*

We hypothesize that behavioral variables such as activity, rumination, eating time, locomotion and lying behavior have differential cross-correlation patterns depending on health status and production levels. In consequence, the objectives of this study were to determine differential cross-correlation patterns between behavioral variables measured by RSD during pre- and post-partum in dairy cows culled due to health problems before 60 days in milk (**DIM**) and in healthy controls. Our secondary objective was to describe pre- and post-partum locomotion behavior in high and low producing cows and the association of lying behavior with milk yield.

## Materials and Methods

*Study animals and housing*

This study was performed between January 13th and July 7th, 2016. The Institutional Animal Care and Use Committee of the Colorado State University (Protocol ID: 16-6704AA) reviewed and approved all procedures related with blood drawing, examination of the reproductive tract, and the use of RSD on the study cows. A retrospective analysis was performed on the animals from the nutritional trial discussed in Chapter I. We evaluated differences in behavioral parameters and energy-related metabolites between animals that left the herd due to health reasons and healthy control animals. The data collection started with a single cohort study group of 202 pregnant dairy cows enrolled at 11 ± 6 d (mean ± standard deviation) prior to the expected calving date. The study group consisted in 147 multiparous (**MP**) cows (parity ≥ 2) and 55 primiparous (**PP**) cows. The housing and management conditions were the same as described in chapter I. Briefly, cows were housed in a common research pen with free stalls, with access to an outdoor patio, and ad-libitum water from automatic drinkers. The milking schedules consisted of three daily milking (07:00, 15:00, and 23:00 h) in a rotatory parlor.

*Study design*

Two different studies were performed based on a retrospective evaluation of behavioral variables measured by RSD associated with health and productive performance. Study 1 consisted of a case control study comparing pre- and post-partum behavioral parameters, and serum metabolites in cows culled before 60 DIM (cases) and in healthy controls. The data collected was used to determine differences in the cross-correlation patterns of behavior parameters such as active time, rumination and eating time according to the case and control status.

On the other hand, study 2 investigated locomotion and lying behaviors of a subset of 30 cows enrolled based on their previous lactation milk yield. The relationships between previous milk yield and locomotion and lying behavior, and current milk yield were evaluated. The data collection started at enrollment for both studies and it followed a prospective schedule (Figure 14) up to 60 DIM.

*Case definition*

In study 1, the retrospective analysis was based on the comparison of cases and controls regarding their behavioral data, milk yield, and serum metabolites. Cases were determined when a study cow left the herd due to health reasons before 60 DIM. On the other hand, eligible healthy control cows were determined if they completed the observation period (150 DIM) in absence of clinical disease, became pregnant and were in or above the group average milk yield. From all eligible control cows, a sampling frame was generated and 30 control cows were randomly selected.

In study 2, 30 MP cows were selected for locomotion and lying behavior evaluation. Cows assessed in the nutritional study in chapter I were classified according to their milk production in their previous lactation. Cows were classified as low (**LP**) or high (**HP**) producing cows if their 305-d milk was below or above 1 standard deviation of the group average. From these subgroups, 15 cows from each producing category were randomly selected to wear the pedometers starting at 15 d prior the expected calving date until 60 DIM (Figure 14).

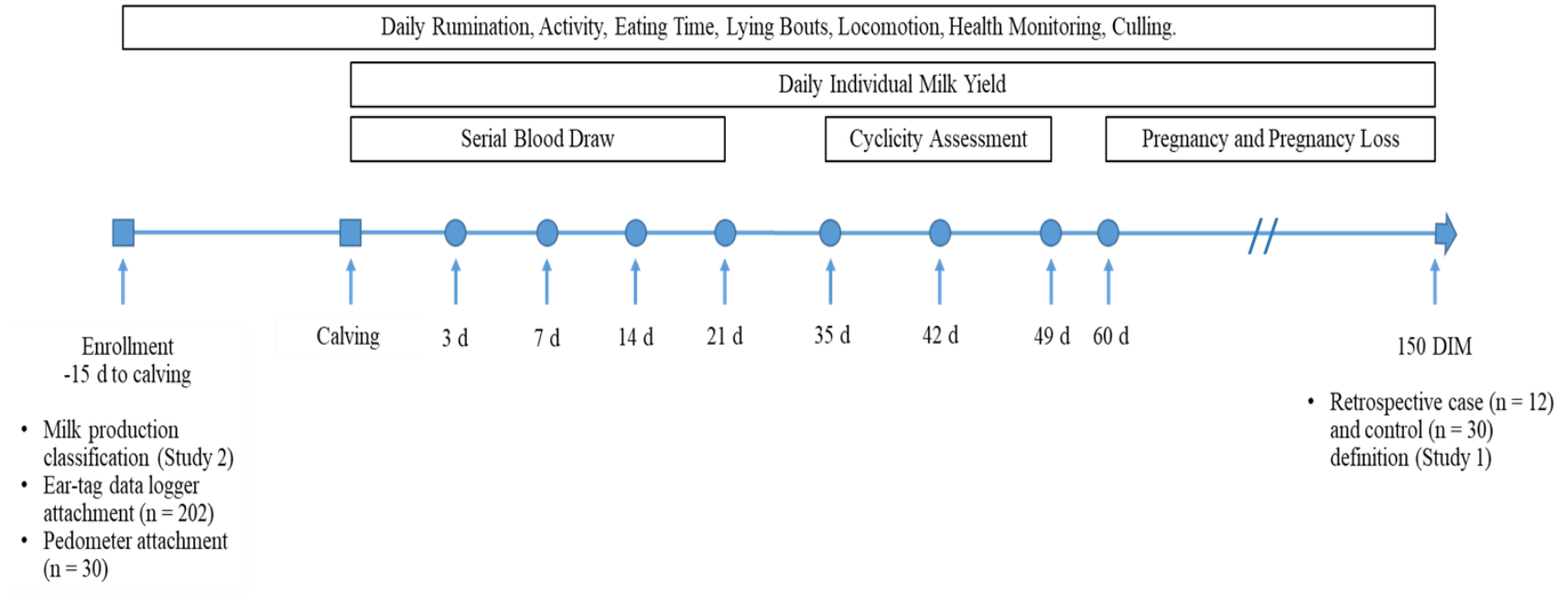



**Figure 14.** Scheme of the outcomes and prospective data collection points in culled and healthy control cows (Study 1) and in low and high producing dairy cows (Study 2).

*Behavioral measurements*

Active, rumination and eating time (min/h) were evaluated in both studies. At 15 d prior to the expected calving date, an ear-tag accelerometer (CowManager SensOor, Agis Automatisering BV, Harmelen, the Netherlands) was attached to the center of the left ear. In addition, in study 2 locomotion and lying behaviors were monitored through pedometers placed around the left metatarsus (Iceqube, IceRobotics, Edinburgh, Scotland, UK). These devices recorded steps (n/d), lying bouts (n/d), and lying time (h/d).

*Milk yield, blood collection and metabolites analysis*

Automatic milking machine software recorded daily milk weights at the three-daily milking. After calving, all cows underwent blood draws from the coccygeal vein at the first of the day after calving and at 3, 7 and 21 DIM. Blood was collected using 18-gauge vacutainer needles and blood collection tubes without anticoagulant (BD Vacutainer, Franklin Lakes, NJ). After collection, the samples were allowed to clot at 4$^{o}$C for one hour and then centrifuged at 2800 rpm for 15 minutes. The supernatant was collected and stored at -20$^{o}$C until laboratory analysis.

Laboratory analyses included determination of glucose, beta-hydroxybutirate (**BHB**), non-esterified fatty acids (**NEFA)** and insulin serum concentrations. Only animals on study 1 were screened for insulin. Glucose (mg/dL) and BHB (mmol/L) were tested using a human hand-held meter (Freestyle Precision Neo, Abbott, Abbott Park, IL) as previously described by Voyvoda and Erdogan (2010). NEFA (mEq/L) were analyzed using an enzymatic colorimetric assay (NEFA-HR (2), Wako Chemicals, Richmond, VA). This assay consisted on the preparations of the provided color reagents A and B and the five standards (NEFA concentrations 0,125, 250, 500, and 1000 uEq/L). In 96-well flat bottom plate, 4 uL of the negative control, standards and sample were pipetted in duplicates. Next, 225 uL of the color reagent A were added to each well and

incubated at 37ºC for 20 minutes. After incubation, 75 uL of the color reagent B were added to each well and incubated another 20 minutes at 37 ºC. Finally, the absorbance of the plate was read in a microplate reader at 560 nm and the NEFA concentration was calculated from the standards using linear regression. Insulin (ug/L) concentrations were determined using a direct sandwich ELISA kit (Mercodia, Uppsala, Sweden).

*Statistical analysis*

In both studies, descriptive analyses of behavioral parameters were performed through plotting and graphical assessment of the daily means over the observation period. Study 1: PROC GLIMMIX of SAS (SAS 9.4, SAS institute Inc., Cary, NC) was used to study overall differences per culling status (outcome) in behavior parameters, as well as milk yield at specific stages prior and during lactation. Activity, rumination, milk yield and locomotion variables were investigated in a model including the binary variable culling status (case or control), parity, DIM, and an interaction term between status and DIM. Differences of serum Glucose, BHB, NEFA and insulin concentrations between cases and controls were analyzed using PROC GLIMMIX by sampling point (calving day, 3, 7, and 21 DIM). This model included status, sampling point and their interaction in the model.

Cross-correlations of behavioral variables by culling status (outcome) were investigated using PROC TIMESERIES of SAS. The time moving variables were created using average accumulation by day (interval time lag) relative to calving day and up to 60 DIM. Plots of behavioral variables were graphically assessed by status classification. Before the CCA, smoothing moving averages (-3t) were calculated (PROC EXPAND) for all behavioral variables to remove white noise. The CCA consisted in bivariate assessment of all behavior by culling status.

In study 2, univariate analysis to evaluate confounding effect of lactation number on the milk category was performed using Chi-square test (PROQ FREQ). Response variables included steps (n/d), lying bouts (n/d), lying time (h/d), and milk yield (kg/d). Explanatory variables included production categories (HP or LP), DIM, and an interaction term between production category and DIM. PROC GLIMMIX was used for the analysis of LSM differences between production categories. Linear regression (PROC REG) was used to evaluate the relationship between lying time and milk yield at 60 DIM in the current lactation. The model included production category, previous lactation number, and the accumulated lying time at 21 DIM. Statistical significance was determined at $P$-value < 0.05 and tendency at $P$-value ≤ 0.15.

**Results and Discussion**

*Study 1*

Study cows included 12 cows (MP = 10; PP = 2) culled due to health reasons and 30 healthy controls cows (MP = 20; PP = 10). Culling reasons included respiratory disease (n = 3), acidosis (n = 2), toxic metritis (n = 2), lameness (n = 2), retained fetal membranes (n = 1), displaced abomasum (n = 1), and heart disease (n = 1).

Involuntary culling of dairy animals is a current concern for dairy farmers and researchers, as reasons for culling decisions are many times related to welfare and health issues reflecting management conditions and the efficiency of the production systems (Compton et al., 2017). Post-partum diseases such as hyperketonemia, retained fetal membranes, displaced abomasum, and uterine infections have been recognized to increase the risk of culling during the first 60 DIM (Seifi et al., 2011; Dubuc et al., 2011; Compton et al., 2017). Although the incidence risk of culling due to low milk production has decreased over the last decades (Comptom et al., 2017), the incidence of culling for reproductive health performance has increased (Dubuc and Denis-

Robichaud, 2017), linked to peripartum metabolic health, which is also connected with milk production (Duffield et al., 2009). These complex interactions make it imperative to understand health indicators for efficient detection of factors associated with culling risk. Traditionally, peripartum diseases such as ketosis, milk fever, retained fetal membranes, metritis, clinical mastitis and displaced abomasum are monitored through serum metabolites such as β-Hydroxybutyrate (**BHB**), non-esterified fatty acids (**NEFA**), calcium, and glucose (Melendez et al., 2009; Seifi et al., 2011), which in elevated levels are associated to culling risk (Overton et al., 2017).

Although predictive associations of pre- and post-partum serum metabolites prior to clinical disease diagnosis have been established (Duffield et al., 2009), the cross-sectional nature of the data, the efficiency of the cut-offs defining diseased animals, and the associated cost, time, and labor of sampling limit their use for disease forecasting.

Overton et al. (2017) recognized that the use of new technologies, which individually monitor behavioral and productive responses, could improve herd health and management by real-time monitoring of health indicators. In this sense, it is plausible to consider that if we can determine temporal associations in cow-behavior to deviations from normality, we could contribute to detect animals with higher culling risk earlier and prevent culling due to health disorders

There is increasing interest in describing patterns of behavioral parameters monitored by RSD in healthy and sick animals. Most available studies performed univariate descriptions of behavioral and physiological parameters. For example, Kovács et al. (2017) described differential patterns of rumination, activity and body temperature in eutotic and dystocic cows, determining that dystocic cows have depressed rumination and body temperature. Additionally, Paudyal et al., 2016 determined that there is differential potential in rumination times in specific health disorders

such as dystocia, clinical ketosis, milk fever, metritis and mastitis and that those fluctuations are affected by climatic conditions. This evidence suggests a predictive potential of behavioral parameters. Nonetheless, there is a lack of research investigating how the dynamic of behavioral patterns measured simultaneously varies by health status, and whether these variables can be used as predictors of others. Additionally, a bivariate approach could be useful to determine which variables are first affected in sick animals.

In our study, active time did not differ by parity ($P = 0.47$) both in cases and controls. This may be because all study cows were subjects to the same group movements and milking schedules. Consequently, parity effect was removed from the model. The model analyzing active time among cases and controls resulted in a significant interaction between culling status and DIM ($P < 0.0001$). Figure 15 shows activity fluctuations during pre- and post-partum days per observation groups. Overall, cows culled before 60 DIM tended to be less active than healthy control cows ($10.1 \pm 0.53$ vs. $11.1 \pm 0.32$ min/h; $P = 0.1$); however, the significant interaction should be further examined to determine specific stages when culling status had significant deviations from healthy control cows. In this sense, Figure 15 shows that 10 days prior parturition cows that would be culled had lower active time compared to cows that would have a better performance or health up to 150 DIM.

Ruminating time represents a very sensitive variable whose fluctuations have been linked to health status and calving behavior (Paudyal et al., 2016; Rutten et al., 2017). Our study agrees with the data presented by Paudyal et al. (2016) where cows affected with peripartum diseases have lower rumination time. Additionally, our analysis on the overall rumination time during the observation period resulted in a significant interaction between culling status and DIM ($P < 0.0001$). Unlike the data presented by Paudyal et al. (2016), we did not observe clear pre-partum

differences in rumination time, which may be due to different sample sizes and study settings. However, we observed that culled cows had a marked drop in rumination time right after calving and that those animals were not able to reach rumination levels as healthy control cows (Figure 16). Overall, cows defined as cases in this study had significantly lower rumination compared to controls (11.7 ± 0.95 vs. 18.6 ± 0.56; $P < 0.0001$).

As rumination time is highly associated with the health status of post-partum dairy cows, further studies associating accumulated ruminating time at specific lactation stages could help to improve health management regarding early interventions and/or culling decisions, and welfare assessment.

Although the effect of parity was not compared between culling status, a tendency was found for parity effect during the study period, where MP cows tended to spend more time ruminating compared to PP cows (16. 13 ± 0.57 vs. 14.2 ± 0.91 min/h; $P = 0.05$).

Eating time differed significantly between cases and controls. A significant interaction between culling status and DIM was found and the fluctuations of eating time are shown in Figure 17. As with active and ruminating time, some evidence of differential pre-partum behavior was observed in eating time, which could represent some value for predictive performance after calving. However, the bigger differences were observed after calving, when cows that would leave the herd due to health disorders had considerable lower daily eating time. Overall, culled cows had lower eating time compared to healthy controls (8.8 ± 1 vs. 15.2 ± 0.6 min/h; $P <0.0001$). A tendency of parity effect on eating time was found ($P = 0.06$). During the observation period MP ate on average 11 ± 0.6 min/h, while PP cows ate 12.96 ± 1 min/h.

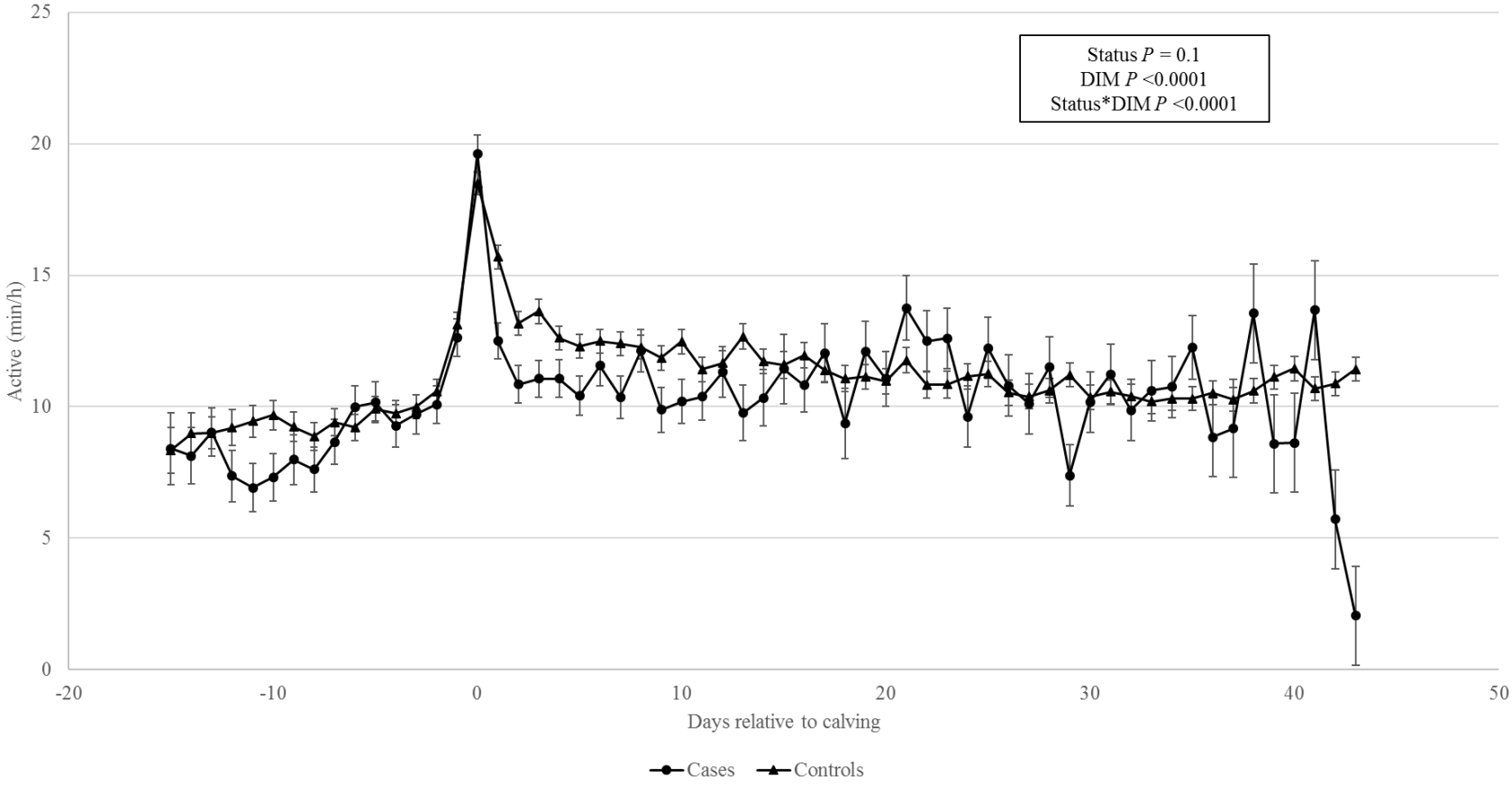



**Figure 15.** Daily average of active time (min/h) relative to calving day (day 0) until 60 DIM in cows culled due to health disorders before 60 DIM (cases; n = 12) and in healthy control cows (n = 30).

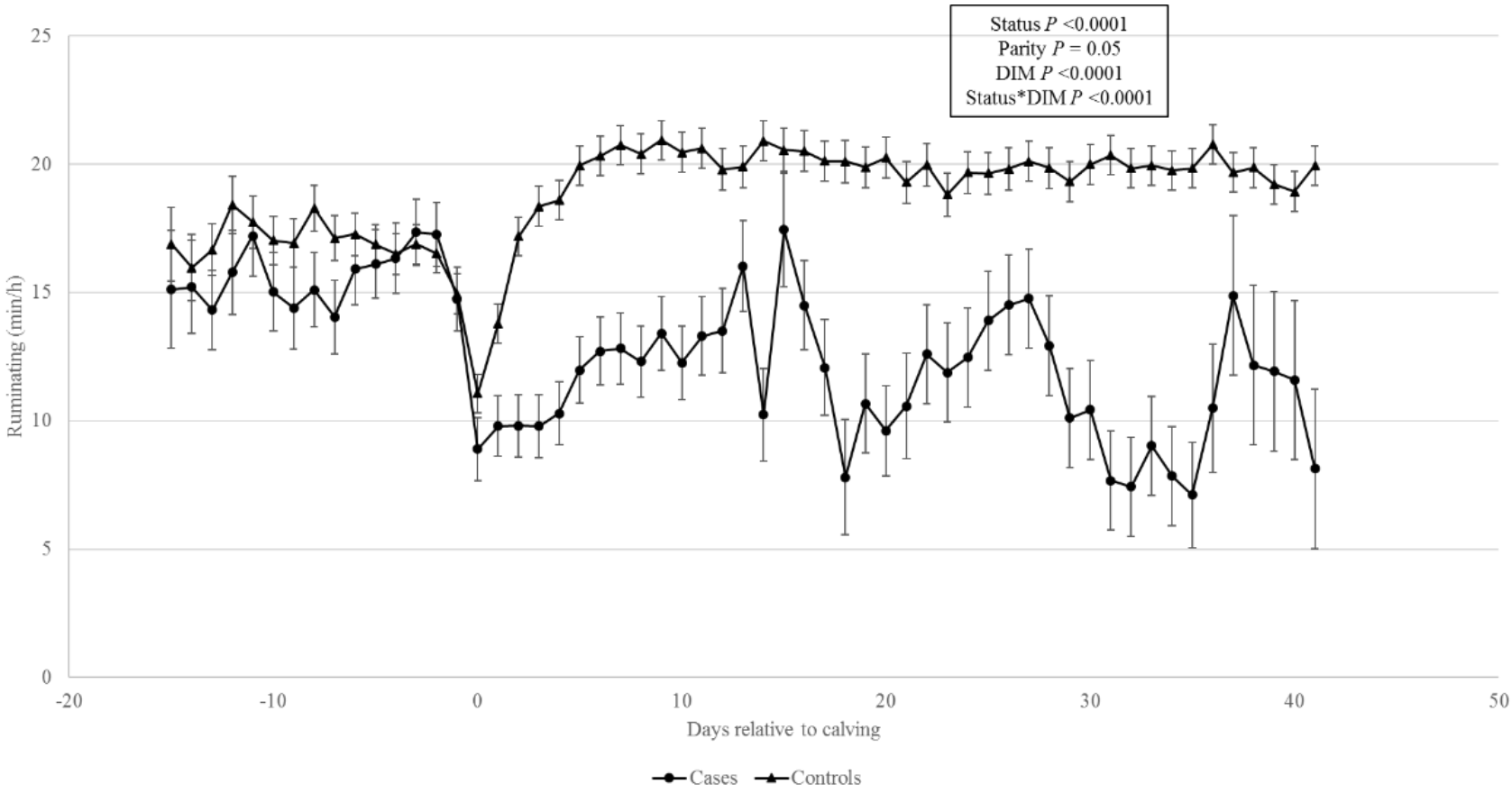



**Figure 16.** Daily average rumination time (min/h) relative to calving day (day 0) until 60 DIM in cases cows (n = 12) versus healthy control cows (n = 30).

Daily milk yield can also be analyzed as a time series variable. Milk production is affected by health status and it is recognized that healthy cows produce more milk than sick cows (Ruprechter et al., 2018) since abrupt reductions in milk yield are linked to ongoing metabolic and inflammatory disorders (Duffield 2009; McArt et al., 2012). In this study milk yield greatly varied between cases and controls. Cows that left the herd before 60 DIM due to health reasons non-related to low production, produced on average 13 kg/d less compared to healthy controls (15.6 ± 2.6 vs. 29.34 ± 1.3 kg/d; $P < 0.0001$). As with active, rumination, and eating time, there was a significant effect between culling status and DIM on milk yield up to 60 DIM. Lactation curves in the cases and controls cows are shown in Figure 18.

Increased levels of BHB and NEFA during post-partum period have been associated with higher culling risk during the first 60 DIM (Seifi et al., 2011; Overton et al., 2017). Carbohydrate metabolism also plays a role in the energy balance adaptation during early post-partum, and it is accepted that dairy cows undergo some extent of insulin resistance during transition (Koster and Opsomer, 2013). However, no associations have been found between glucose concentrations and the risk of culling at 60 DIM (Seifi et al., 2011). The interaction between lipid and carbohydrate metabolites had been recognized to play a key role in the understanding of metabolic diseases during transition (Koster and Opsomer, 2013). In this study, we evaluated the association between BHB, NEFA, glucose and insulin concentrations and the culling status through sequential blood draws during the first 21 DIM. A summary of the concentrations of metabolites evaluated in this study is presented in Table 10. Additionally, distributions of the metabolites evaluated at calving day, 3, 7 and 21 DIM per culling status (cases and controls) are shown in Figures 19 to 22.

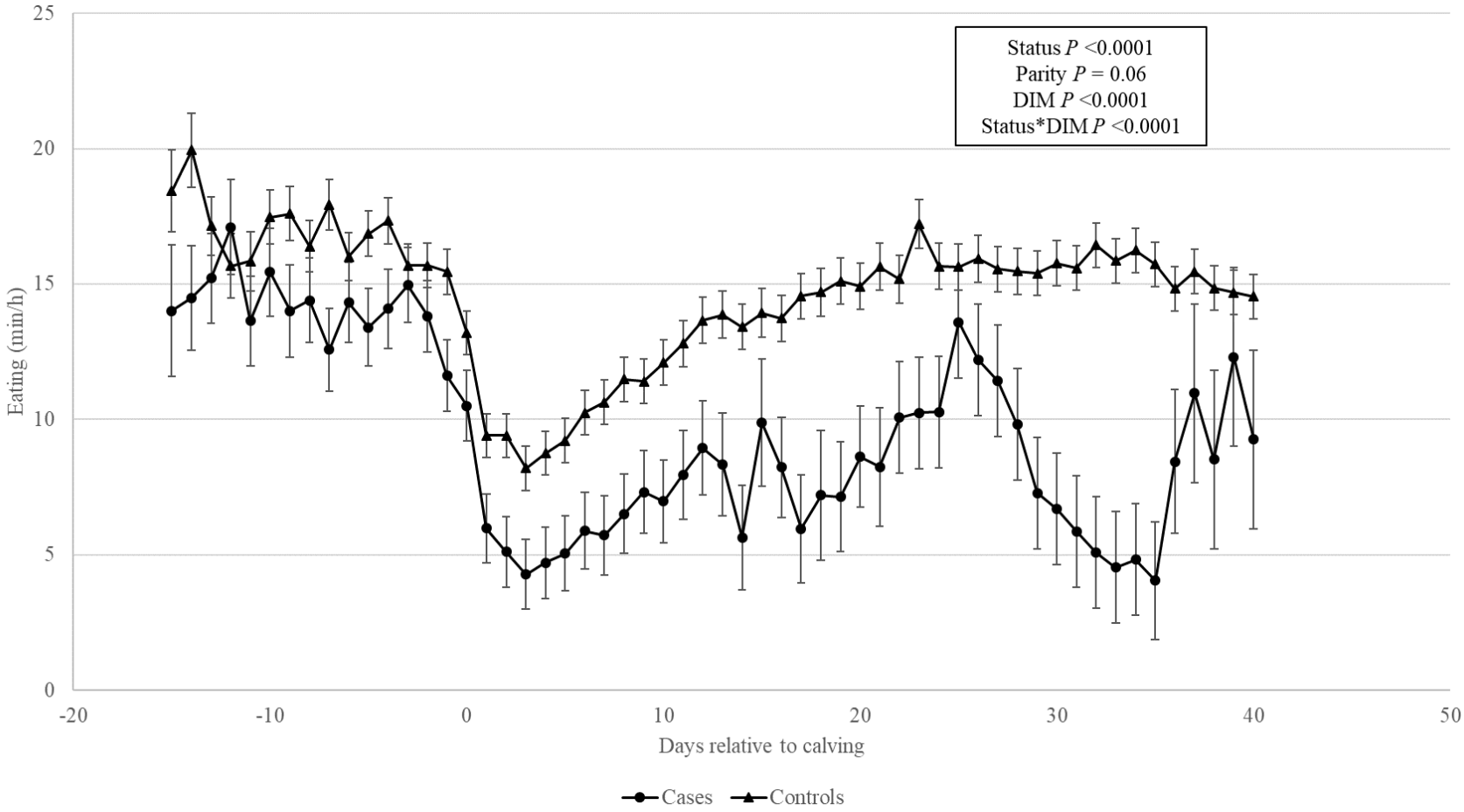



**Figure 17.** Daily average eating time (min/h) relative to calving day (day 0) until 40 DIM in cases cows (n = 12) versus healthy control cows (n = 30).

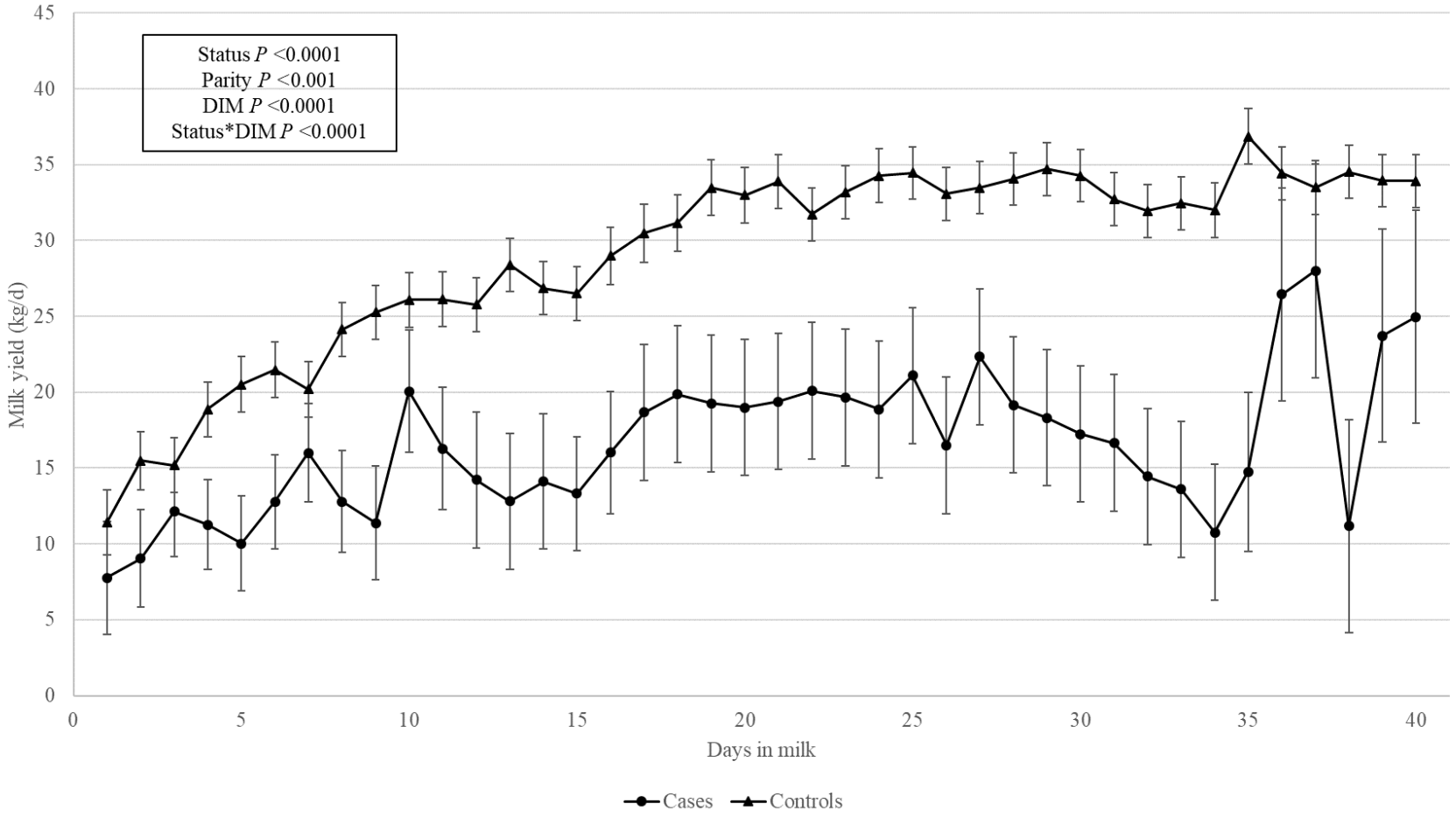



**Figure 18.** Daily milk yield (kg/d) in cows culled (cases, n = 12) before 40 DIM due to health reasons and healthy controls cows (n = 30).

Overall, there was a significant association between culling status and BHB concentration. Cows that left the herd before 60 DIM had higher concentration of BHB compared to healthy control cows (2.27 ± 0.31 vs. 1.42 ± 0.17 mmol/L; $P = 0.02$). Regarding differences at the sampling times, BHB concentrations within 24 h after calving did not differ among cases and controls (1.16 ± 0.36 vs. 1.19 ± 0.22 mmol/L; $P = 0.9$). At 3 DIM both groups had an increment in BHB concentration relative to calving day, which was more marked in culled cows compared to healthy controls (2.23 ± 0.38 vs. 1.7 ± 0.23 mmol/L). However, no statistical difference was observed ($P = 0.19$).

At 7 DIM, cases had significantly higher BHB concentrations than controls (3.1 ± 0.41 vs. 1.8 ± 0.23 mmol/L; $P < 0.001$). Finally, at 21 DIM BHB concentrations were also higher in cases than in controls (2.65 ± 0.61 vs. 1.1 ± 0.22 mmol/L; $P = 0.02$). These results agree with most published literature relating high BHB concentration with culling (Seifi et al., 2011; Overton et al., 2017).

Distribution of NEFA concentrations between cases and controls are presented in Figure 20. Overall, there was no difference between cases and healthy controls in NEFA concentration (0.38 ± 0.04 vs. 0.32 ± 0.02; $P = 0.18$). Samples collected during on calving day did not show differences for cases vs. control cows (0.31 ± 0.04 vs. 0.34 ± 0.03 mEq/L; $P = 0.6$). At 3 DIM, cases had significantly higher NEFA concentration than healthy control cows (0.45 ± 0.05 vs. 0.33 ± 0.03 mEq/L; $P = 0.03$). No differences were found in NEFA concentrations among cases and control cows at 7 and 21 DIM (0.38 ± 0.05 vs. 0.34 ± 0.03 mEq/L; $P = 0.5$, and 0.36 ± 0.08 vs. 0.26 ± 0.03 mEq/L; $P = 0.23$).

**Table 10.** Comparison of the concentrations of glucose, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA) insulin within 24 h after calving, and at 3, 7, and 21 DIM in cows culled before 60 DIM (cases) and healthy control cows

| | Glucose (mg/dL) | | | | | BHB (mmol/L) | | | | | NEFA (mEq/L) | | | | | Insulin (ug/L) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sampling points | Case | SE | Control | SE | *P*-value | Case | SE | Control | SE | *P*-value | Case | SE | Control | SE | *P*-value | Case | SE | Control | SE | *P*-value |
| Calving | 87 | 9.6 | 91.3 | 6 | 0.7 | 1.15 | 0.36 | 1.19 | 0.23 | 0.93 | 0.31 | 0.04 | 0.34 | 0.03 | 0.6 | | | | | |
| 3 DIM | 75.8 | 10.4 | 72.4 | 6.1 | 0.29 | 2.23 | 0.38 | 1.65 | 0.23 | 0.19 | 0.45 | 0.05 | 0.33 | 0.03 | 0.03 | 0.16 | 0.04 | 0.23 | 0.02 | 0.13 |
| 7 DIM | 93.3 | 11.6 | 87.7 | 6.1 | 0.23 | 3.1 | 0.4 | 1.75 | 0.23 | <0.001 | 0.38 | 0.05 | 0.34 | 0.03 | 0.5 | 0.1 | 0.04 | 0.13 | 0.02 | 0.6 |
| 21 DIM | 61.1 | 18.5 | 64.3 | 6 | 0.81 | 2.64 | 0.6 | 1.1 | 0.23 | 0.02 | 0.36 | 0.08 | 0.26 | 0.03 | 0.23 | 0.12 | 0.06 | 0.16 | 0.02 | 0.6 |

Serum glucose has not been used as a reliable marker for metabolic status and it is not associated with displaced abomasum, clinical ketosis, and culling (Seifi et al., 2011). In this study there were no overall or temporal differences between study groups and sampling points in glucose concentrations. On the other hand, insulin has been pointed out as a valuable metabolic marker to understand the metabolic syndrome that most transition dairy cows undergo.

There is not enough evidence to determine threshold levels for insulin to define pathological concentrations in dairy cows but it has been suggested that insulin plays a pivotal role in the glucose metabolism of dairy cows and it responds to levels of BHB and NEFA (Koster and Opsomer, 2013). In our study, we did not observe differences in the insulin concentrations among cases and controls; however, from the distribution of insulin concentrations (Figure 9) it can be observed that cows that would leave the herd due to health reasons had lower median concentrations compared to healthy controls. This area should be subject to intensive research to attain such conclusions.

As discussed to this point, all behavioral parameters and the metabolites show relationship with culling status. Following this evidence, we were interested in investigating temporal relationships between behavior variables and production, and whether these associations had differential patterns in culled and healthy control cows. The first step in the analysis of time series related to a singular period is the graphical assessment of their fluctuations over time. Figure 23a shows the daily average of the behavioral variables (activity, rumination and eating time) plus daily milk yield in all the study cows (N = 42). To determine serial correlations between variables collected sequentially overtime, the application of smoothers that remove white noise is needed (Shumway and Stoffer, 2016).

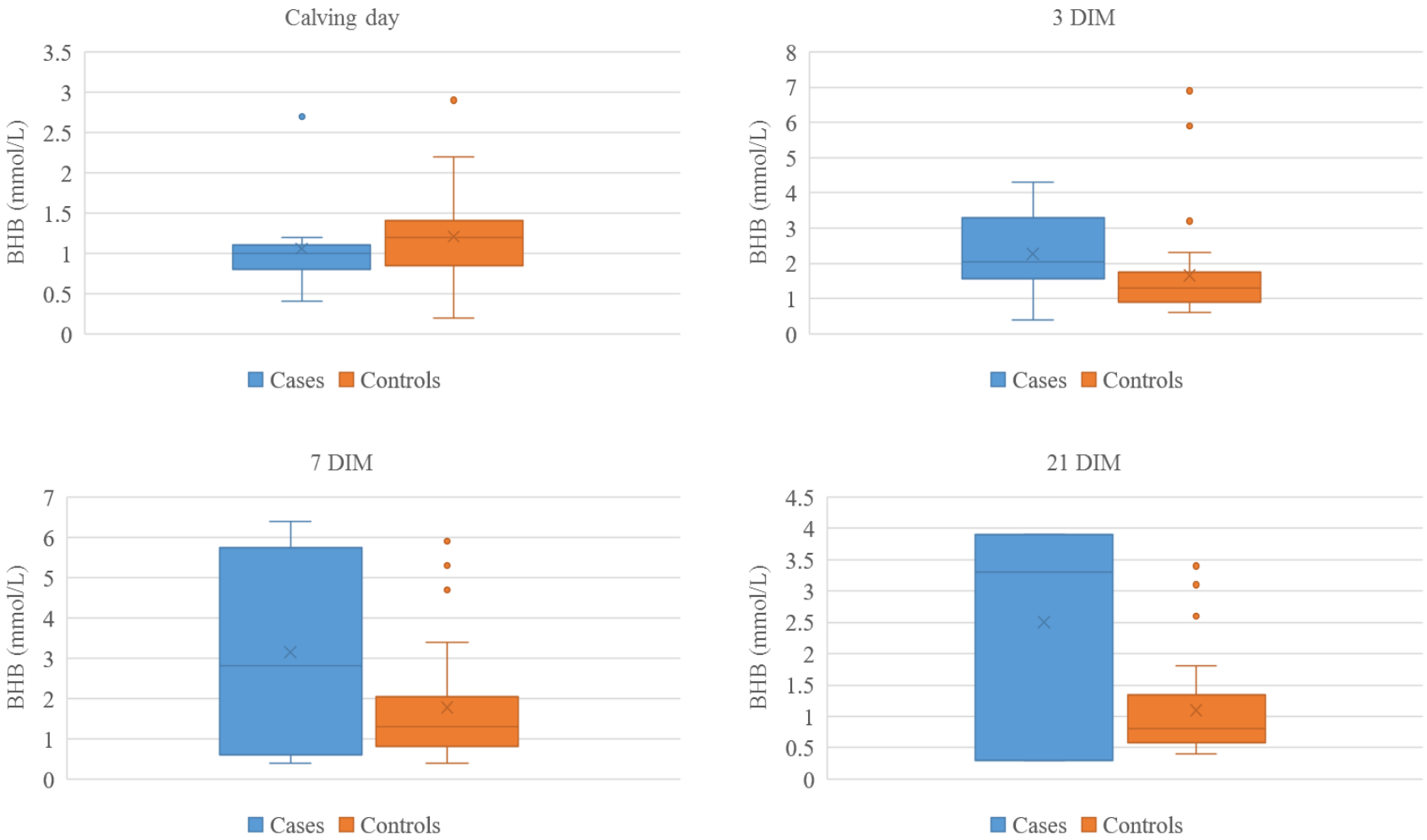



**Figure 19.** Distribution of BHB concentrations at calving day, 3, 7 and 21 DIM in cows culled before 60 DIM due to health reasons (cases, n = 12) and in healthy control cows (n = 30). There was no statistical difference between study group means

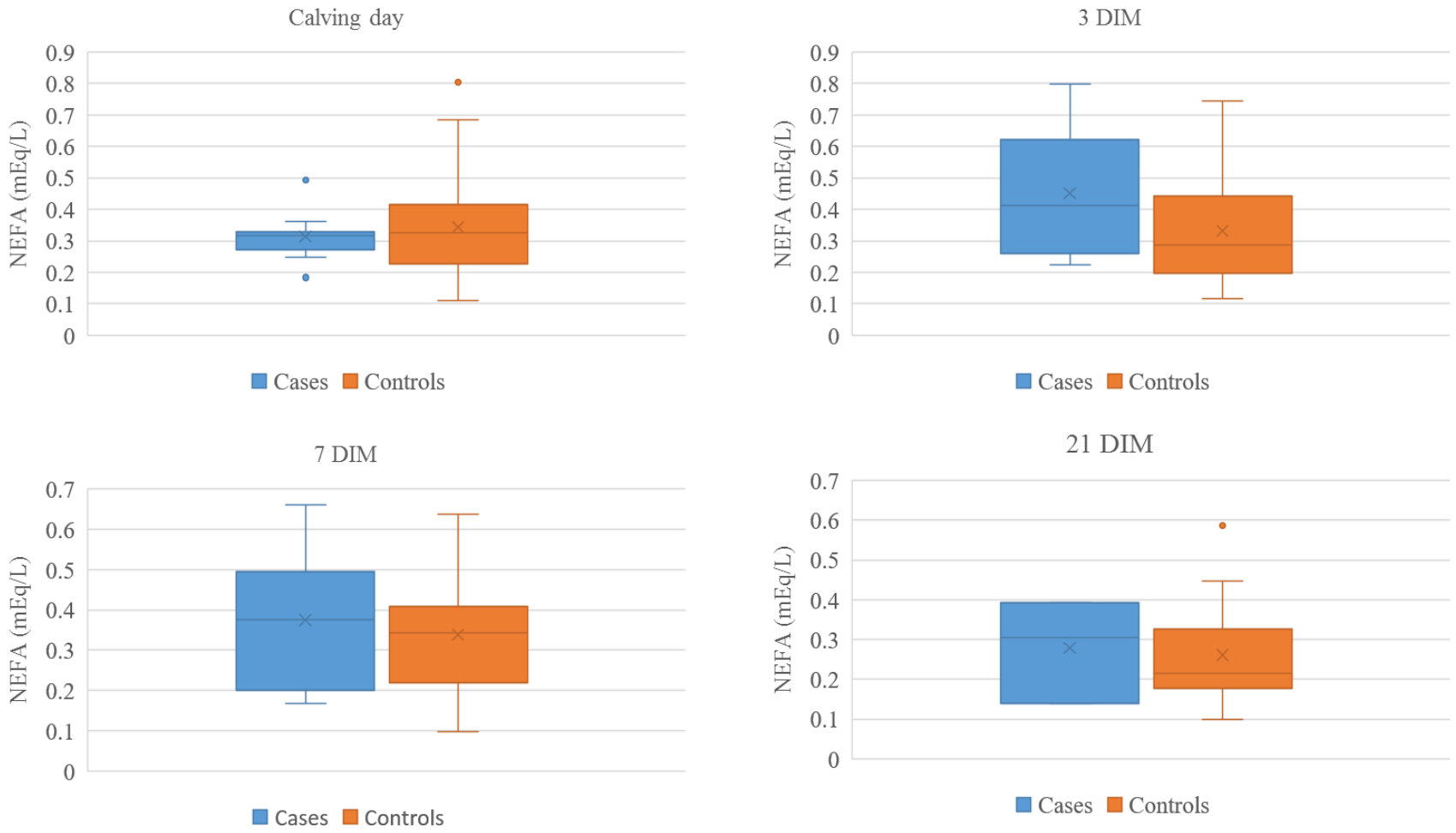



**Figure 20.** Distribution of NEFA concentrations at calving day, 3, 7 and 21 DIM in cows culled before 60 DIM due to health reasons (cases, n = 12) and in healthy control cows (n = 30). There was no statistical difference between study group means

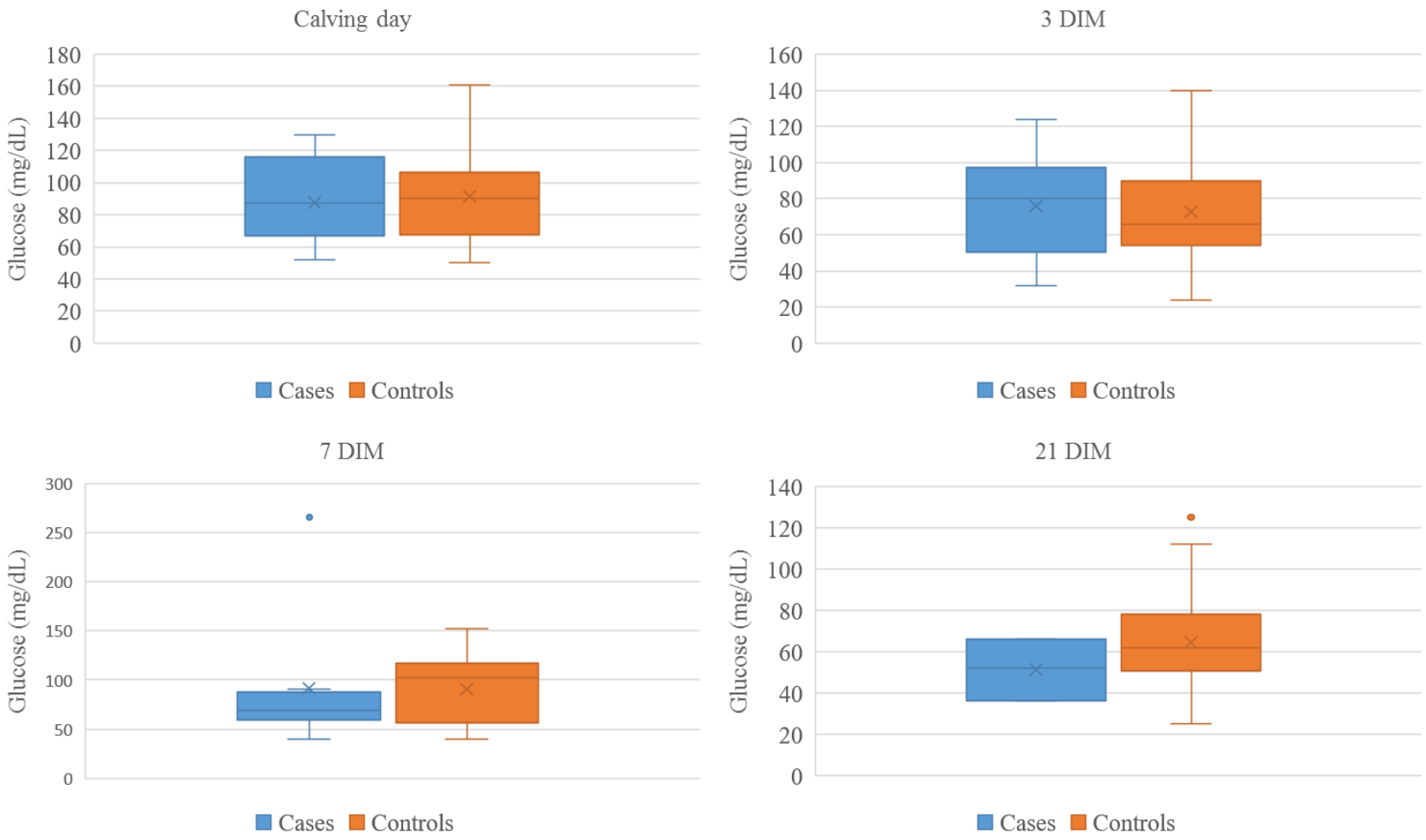



**Figure 21.** Distribution of glucose concentrations at calving day, 3, 7 and 21 DIM in cows culled before 60 DIM due to health reasons (cases, n = 12) and in healthy control cows (n = 30). There was no statistical difference between study group means

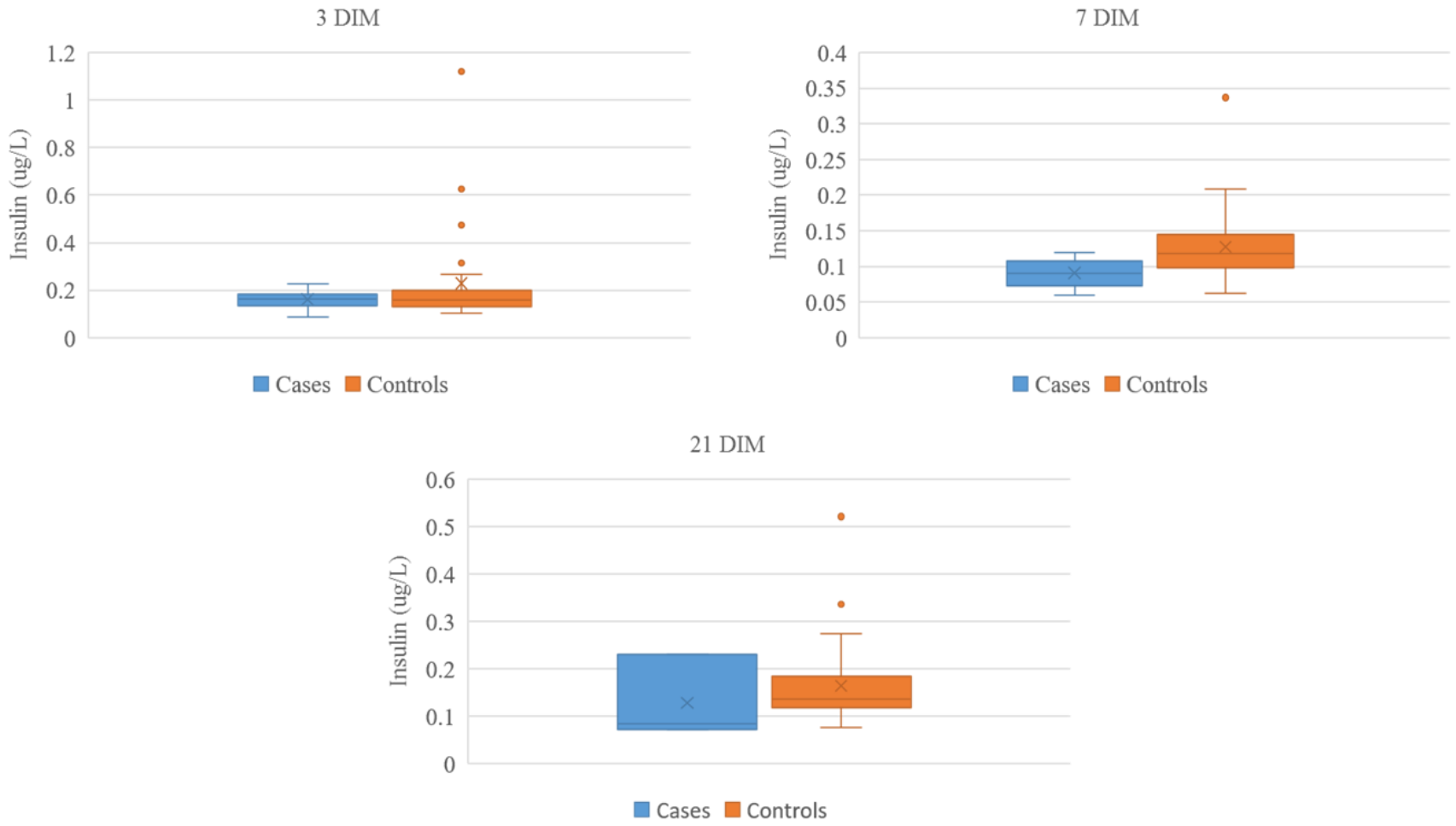



**Figure 22.** Distribution insulin concentrations (ug/L) at calving day, 3, 7 and 21 DIM in cows culled before 60 DIM due to health reasons (cases, n = 12) and in healthy control cows (n =30). There was no statistical difference between study group means

Figure 23b shows smoothed series after the use of a moving average, considering current and two previous values. Moving averages were generated for all cross-series plots per culling status. Only transformed series are shown.

Cross-correlation analysis studies the relationship between two time series related to past lags (past observations). Sample cross-correlation can be graphically assessed to search leading or lagging relstions between two time series (Shumway and Stoffer, 2016). In the idea of exploring behavioral fluctuation from all study cows (N = 42), simple correlations, magnitude and direction of the cross-correlations of significant time lags between the pairs of behavioral and milk production variables were calculated. The simple correlation during the observation period is represented by the value of the time lag 0, which consider all observations.

The simple correlation between rumination and activity showed a negative linear correlation of -0.38 ($P$ = 0.004). However, this value does not include information about time fluctuations, trends and relationships between two variables that may occur during an observation period. From the cross-series plot (**CSP**) between rumination and activity relative to calving date (Figure 24), activity showed earlier increases as calving date approached compared to rumination. Additionally, changes in activity were assessed by the cross-correlation functions since positive time lags had significant cross-correlation, which is commonly observed when one variable (rumination) is lagged by changes in the other (activity). In other words, activity could be used as predictor of rumination during the peripartum period. Significant time lags, which are defined by day interval, were observed between lags 6 and 16. Commonly, the greatest cross-correlation value is used as an estimate of the relationship between two time moving variables. In the case of the rumination and activity relationship, this occurred at lag 9 with a cross-correlation of 0.63 ($P$ <0.0001).

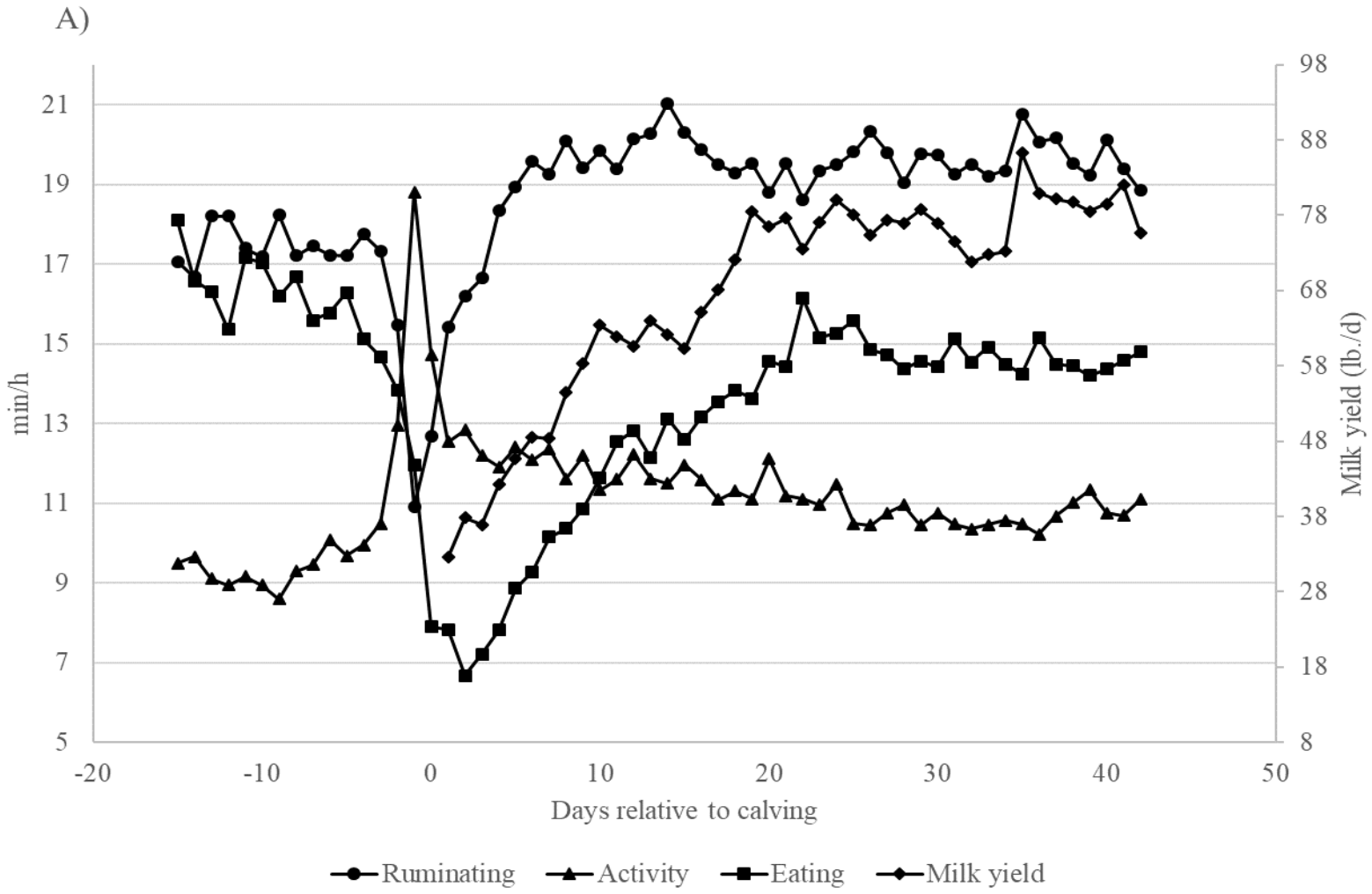
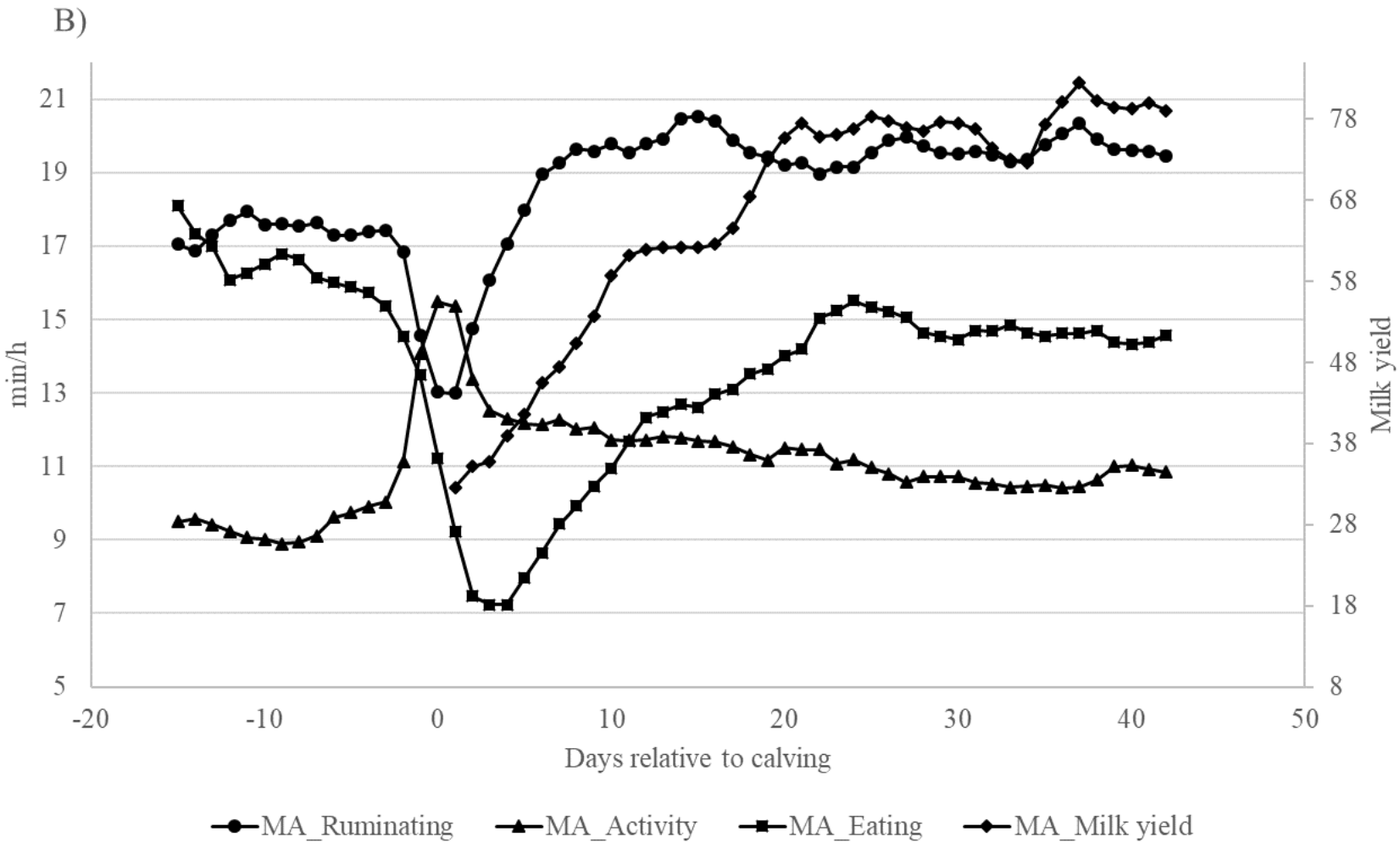



**Figure 23.** Fluctuation of the behavioral parameters (active, ruminating and eating time) and daily milk yield in all study cows (N = 42) relative to calving day (day 0). Graph A, shows time series variables without smoothing moving average (MA), whereas graph B (next page) shows the same variables after MA transformation


B)
21
19
17
15
13
11
9
7
5
min/h
78
68
58
48
38
28
18
8
Milk yield
-20
-10
0
10
20
30
40
50
Days relative to calving
MA_Ruminating
MA_Activity
MA_Eating
MA_Milk yield

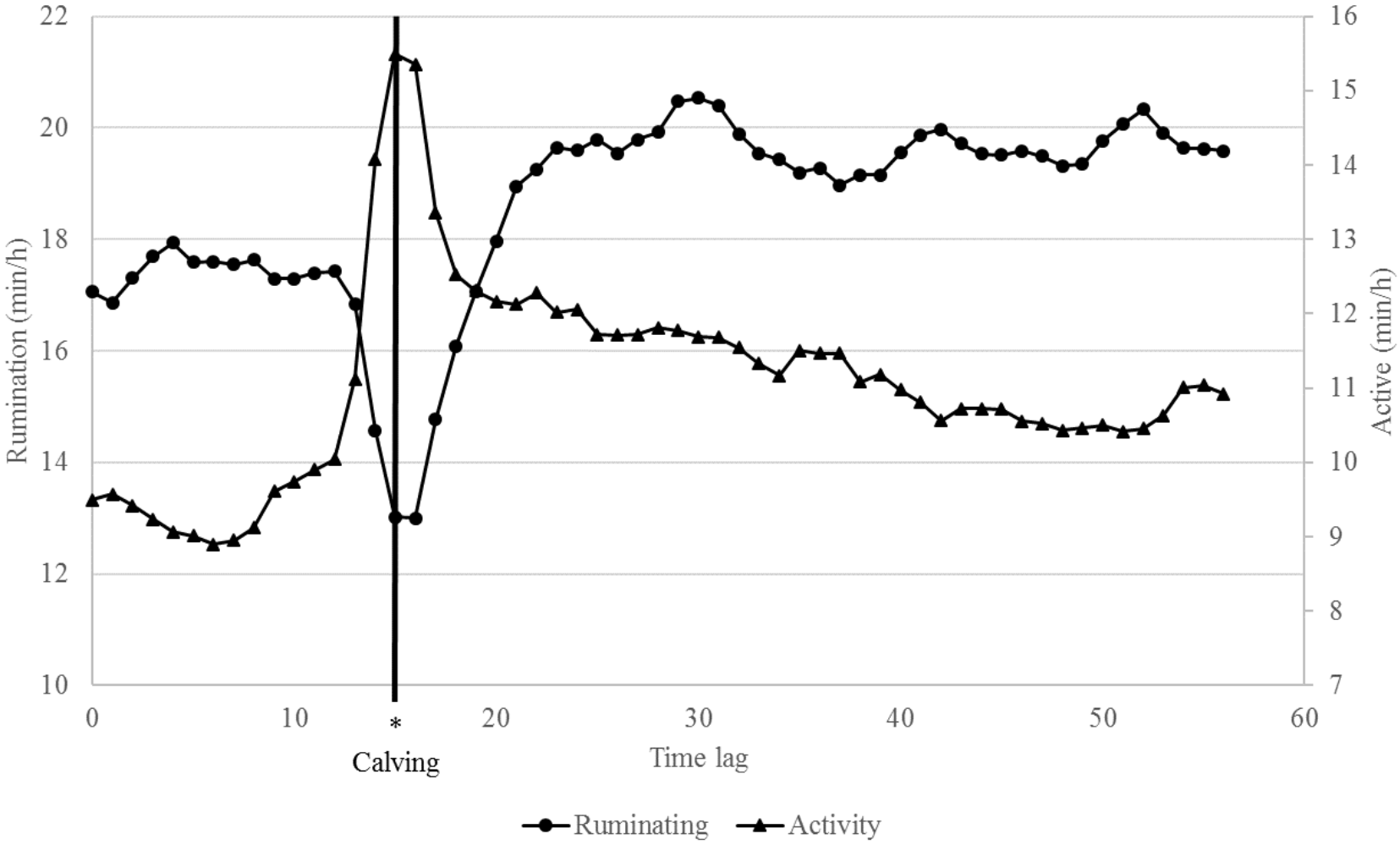



**Figure 24.** Cross-series plot between rumination and activity obtained from all study cows (N =42) from 15 days pre-partum until 45 DIM. Time lags represent 1 d average of rumination and activity

In simpler words, this implies that when deviations in activity over the average occur, deviations in the rumination average will occur about 9 days later in the same direction due to the positive sign of the significant cross-correlation. This finding suggests a time relationship between rumination and activity in the data collected from the study animals.

Cross-series plots and cross-correlation functions of the other behavioral parameters and milk yield are presented in Figures 25 to 26 and Table 11, respectively. Regardless of culling status, the temporal relationship between rumination and eating time did not show a simple linear correlation (r = 0.2; *P* = 0.12; Table 11). The CSP between rumination and eating time (Figure 25) shows similar curve fluctuations over time, which may be explained because of their physiological relationship. On the other hand, the cross-correlation functions showed a significant time relationship, where fluctuation in the time ruminating leaded changes in eating time. The greatest and significant lag was -5 with a cross-correlation estimate of 0.56 (*P* <0.0001). Therefore, it is suggested that changes in eating time above the average are related to changes in rumination that occurred 5 d before. This supports the idea that rumination might show earlier deviations associated to calving or disease compared to eating behavior.

The temporal relationship between activity and eating time was evaluated. The simple linear correlation was -0.8 (*P* < 0.0001). In the same line, the cross-correlation functions showed a strong time relationship where activity leaded eating time fluctuations (Table 11). In other words, activity might be considered a predictor of eating time. The greatest cross-correlation when these two time series variables were evaluated was reached at lag -2 (-0.82; *P* < 0.0001), therefore, changes in activity will lead in changes in eating time in opposite direction (Figure 26).

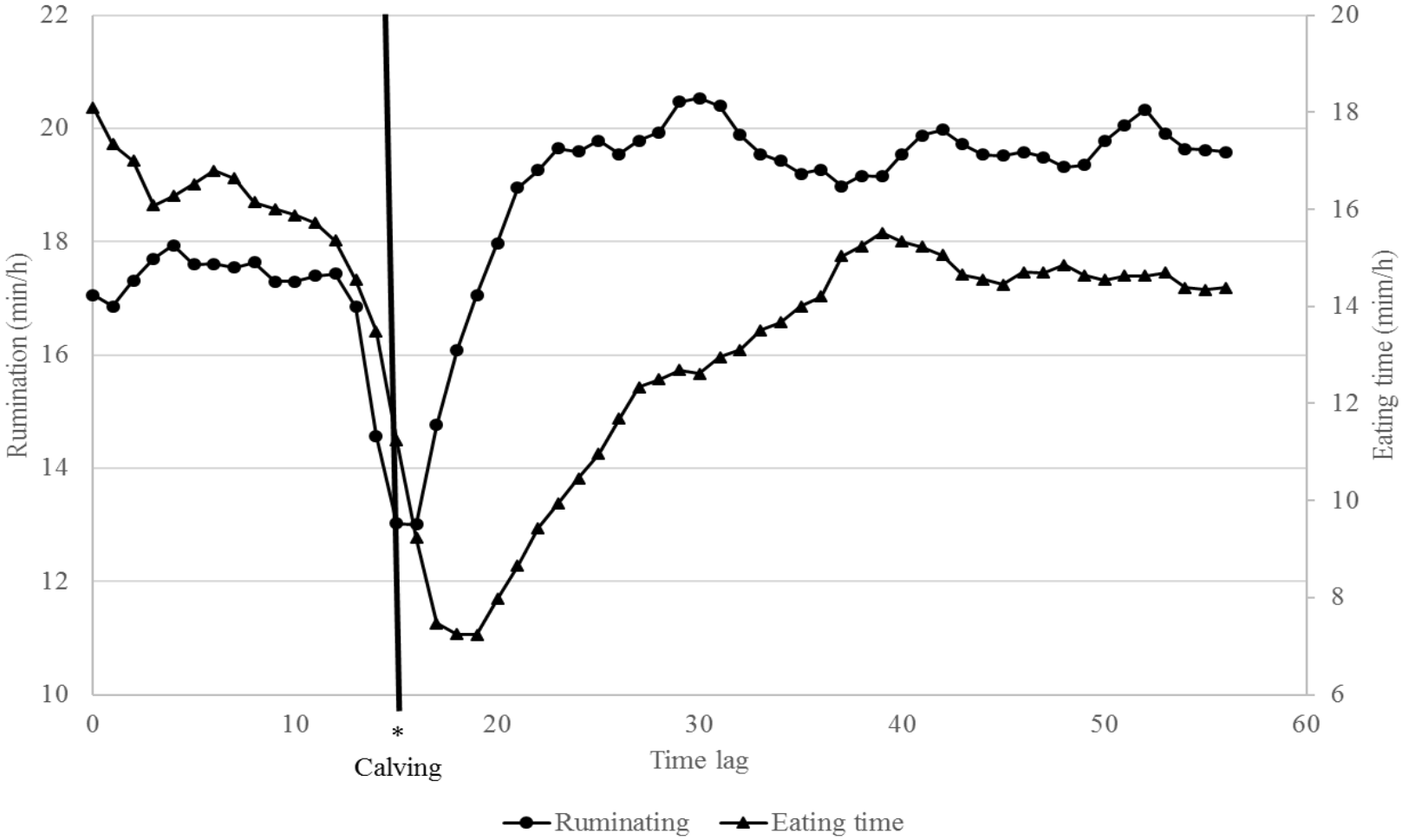



**Figure 25.** Cross-series plot between rumination and eating time obtained from all study cows (N =42) from 15 days pre-partum until 45 DIM. Time lags represent 1 d average of rumination and activity.

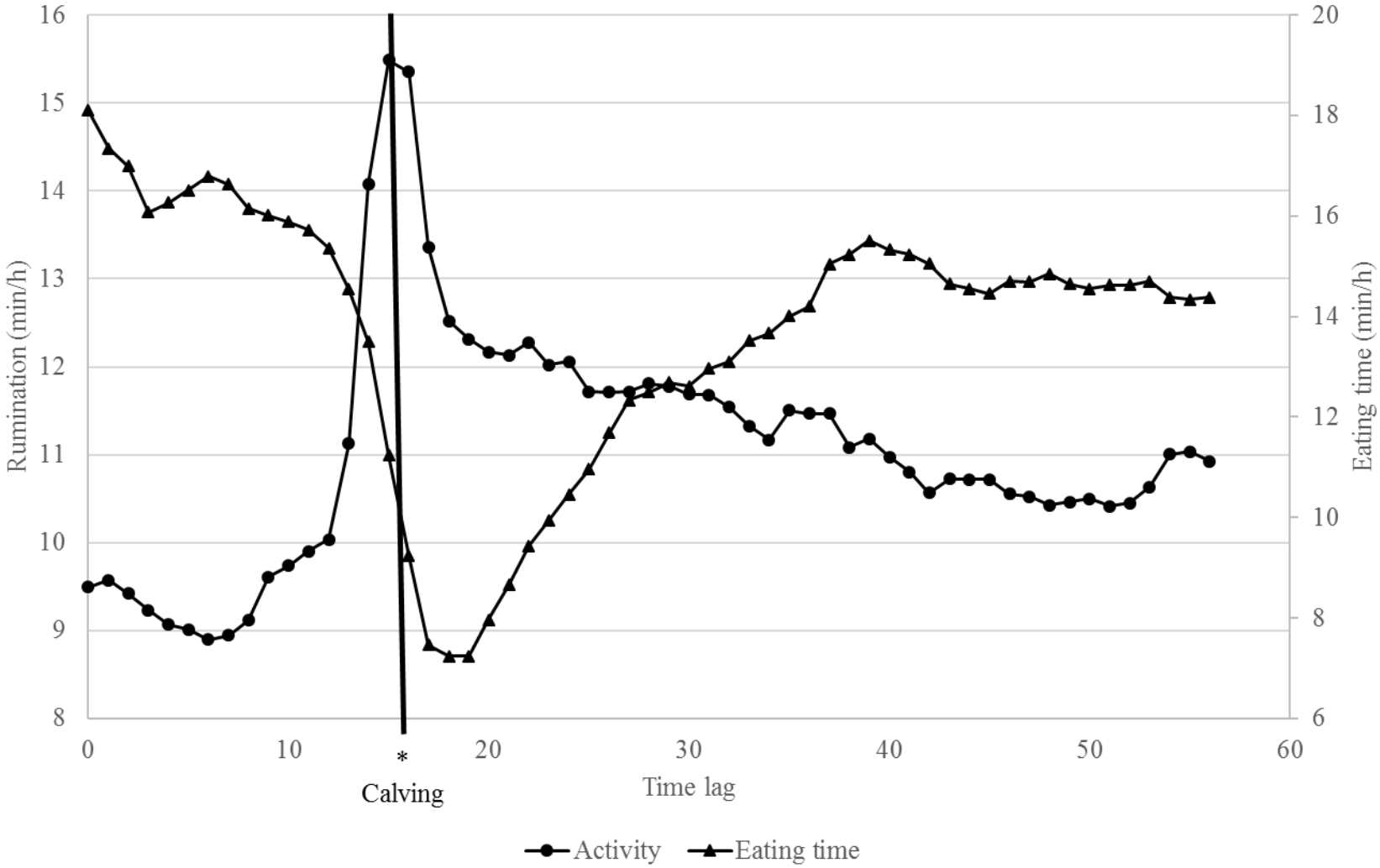



**Figure 26.** Cross-series plot between rumination and eating time obtained from all study cows (N =42) from 15 days pre-partum until 45 DIM. Time lags represent 1 d average of rumination and activity.

The objective of the analysis without considering the culling status stratification was to show how behavior variables fluctuate in a group of lactating cows and to help interpreting the information obtained from CCA. A similar analysis was subsequently performed including the culling status category. Cross-series plots by culling status show clear differential patterns between the behavioral parameters and milk yield. The observed differences resulted in a clear disparity in the time relationship when the behavioral and milk yield variables were evaluated by culling status. For example, in culled cows the simple correlation between rumination and activity was slightly stronger compared to the correlation calculated using the data without the culling status strata (Table 11). Conversely, the simple correlation was not found in healthy controls (r = -0.15; *P* = 0.25). Temporal relationship between rumination and activity had also different patterns when comparing cases and controls. While cases had the greatest cross-correlation at lag 1 (-0.51; *P* <0.0001), controls had relationships patterns similar to those observed using data without culling status (Table 11). Controls had the greatest cross-correlation at lag 7 (0.62; *P* <0.0001). This implies that cows that would be culled before 60 DIM had a different associations between rumination and activity regarding the magnitude, and direction of cross-correlations and time lags. This was also the case for the other bivariate temporal relationships presented in Table 11, where cases had a specific pattern of cross-correlations.

We observed lower cross-correlations between activity and eating time for both cases and controls. Additionally, those relationships were observed at the same lags. Nonetheless, cases had weaker cross-correlation between activity and eating time regarding simple correlation and cross-correlation compared to controls (Table 11).

**Table 11.** Simple linear correlation and cross-correlation estimates of behavioral parameters (active, ruminating and eating time) and milk yield in study cows (N = 2) and in culled cows before 60 DIM (cases) and healthy control cows. Time lags of the greatest cross-correlations and *P*-values are included

| | All data (N = 42) | | | | | Cases (n = 12) | | | | | Controls (n = 30) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Varibles | Simple Correlation | *P*-value | Cross-corelation | Lag | *P*-value | Simple Correlation | *P*-value | Cross-correlation | Lag | *P*-value | Simple Correlation | *P*-value | Cross-correlation | Lag | *P*-value |
| Rumination / Activity | -0.37 | 0.004 | 0.62 | 9 | <0.0001 | -0.47 | <0.0001 | -0.52 | 1 | <0.0001 | -0.15 | 0.25 | 0.66 | 7 | <0.0001 |
| Rumination / Eating time | 0.2 | 0.12 | -0.54 | 9 | <0.0001 | 0.82 | <0.0001 | 0.77 | -1 | <0.0001 | -0.15 | 0.23 | -0.58 | 6 | <0.0001 |
| Activity / Eating time | -0.76 | <0.0001 | 0.84 | -2 | <0.0001 | -0.51 | <0.0001 | -0.58 | -2 | <0.0001 | -0.8 | <0.0001 | -0.86 | -2 | <0.0001 |
| Rumination / Milk yield | 0.69 | <0.0001 | 0.65 | -1 | <0.0001 | 0.19 | 0.2 | 0.43 | -11 | 0.04 | 0.62 | <0.0001 | 0.59 | -1 | <0.0001 |
| Activity / Milk yield | -0.87 | <0.0001 | -0.8 | -1 | <0.0001 | -0.38 | 0.015 | -0.26 | -8 | 0.09 | -0.94 | <0.0001 | -0.86 | -1 | <0.0001 |
| Eating time / Milk yield | 0.96 | <0.0001 | 0.94 | 1 | <0.0001 | 0.6 | <0.0001 | 0.71 | 1 | <0.0001 | 0.89 | <0.0001 | 0.94 | 1 | <0.0001 |

This information suggests that, it is possible to learn how cows that will have impaired health and performance during the first 60 DIM behave regarding rumination, activity, eating time and milk yield even before the algorithms used by RSD can detect an ongoing disease.

For the CCA, it is important to detrend the time moving variables by using smoothers to remove random noise in the time series. Although it was possible to draw conclusions from the data around calving, as calving approached all behavior parameters showed a clear trend associated with calving date (Figure 23). For this, the time relationships were studied separately before and after calving. In the case of milk yield, and for obvious reasons, the analysis considered only post-partum data.

Temporal relationship between rumination and milk yield was similar between healthy control cows and the overall population of study cows, where changes in rumination led to changes in milk yield. In healthy controls, a positive association at time lag 1 characterized this association implying that changes in rumination are reflected in changes in milk yield one day later in the same direction. On the other hand, culled cows had not simple correlation between these two variables (0.19; $P = 0.2$. Table 11) and there was no time relationship because of the differential behavior of the curves in the CCP (Figure 27). Other relationships where milk yield was involved showed a differential association by culling status (Table 11). For instance, healthy controls always had a similar pattern of correlations to those found when all study cows were analyzed.

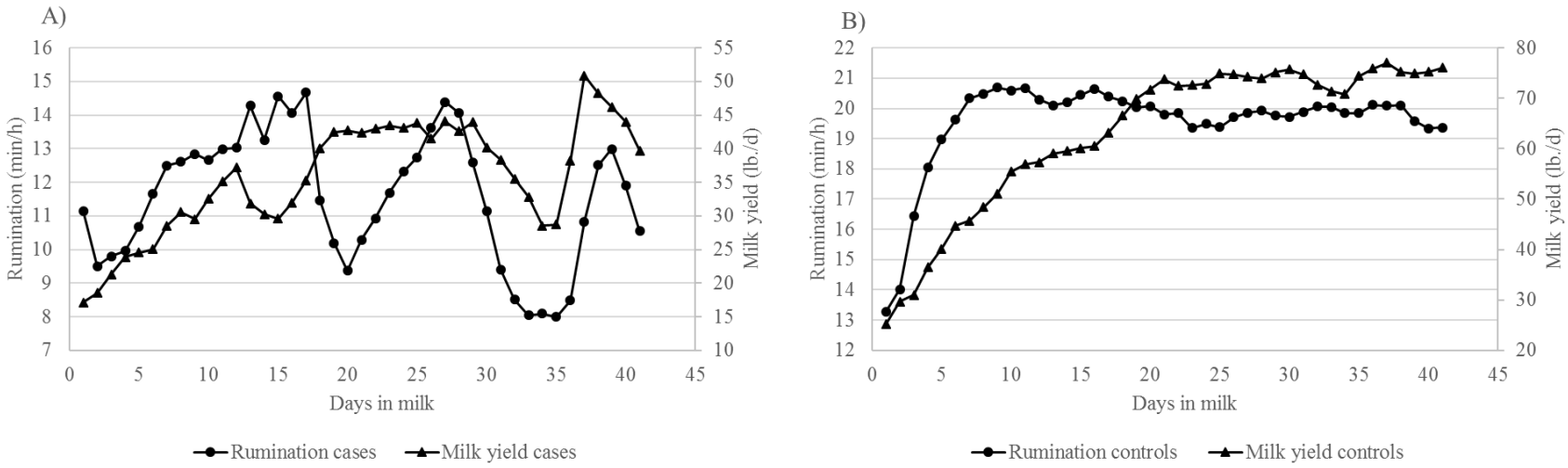



**Figure 27.** Cross series plots of the rumination and milk yield of cows classified as cases (A), and healthy controls (B). Plot A, did not result neither in simple correlation nor time relationship whereas plot B had significant simple correlation 0.62 ($P$ <0.0001) and time relationship at time lag -1 (0.59; $P$ <0.0001)

Results of the CCA stratified by pre- and post-calving data from cases and control cows are shown in Table 12. Cows that would be culled from the herd showed a differential pattern of cross-correlations between the studied time series either at pre- and port-calving stages. In cases, rumination and activity were cross-correlated during pre-partum but not in post-partum (Figure 28, Table 12), while in healthy controls these variables showed independency during pre-partum. In cases during pre-partum, rumination and eating time had a positive cross-correlation, where fluctuations of rumination leaded changes in eating time 6 d later (Figure 29). Conversely, healthy cows had a negative simple correlation and cross-correlations, where eating time was the leading variable at lag 1 (Table 12). During pre-partum, the relationship between activity and eating time resulted significant only in cases (-0.63; $P$ <0.01) at lag 2, whereas no association was found between activity and eating time in control during the pre-calving period (Figure 30, Table 12).

In a similar way, the post-postpartum period had different time relationships when cases or controls were analyzed. Rumination and activity were not related to each other in cases (simple correlation, r = -0.1; $P$ = 0.53) and there was a weak cross-correlation (0.33) at lag 6 (Table 12). On the other hand, there was a clear pattern of rumination and activity in healthy controls characterized by the decrease of activity right after calving and an increase of rumination during the first week post-partum. These curves yielded a negative simple correlation of -0.77 ($P$ <0.0001) and a cross-correlation of -0.63 ($P$ <0.0001) along with activity leading changes of rumination 1 d later (Table 12).

Between cases and controls, temporal relationship between rumination and eating time had the same magnitude regarding the cross-correlation values but with opposite direction. While cases had a negative cross-correlation with eating time leading rumination, controls had positive correlation with rumination leading fluctuations in eating time.

This might be associated to impaired rumination function after calving in cases where increments in the time eating are not necessarily correlated to the time that sick cows spent ruminating.

After calving, the temporal relationship between activity and eating time reached before calving was lost in cases due to a non-significant simple correlation and cross-correlation (Table 12). On the other hand, in healthy controls, these variables resulted correlated after calving (simple correlation: -0.75; $P$ <0.0001; cross-correlation: -0.63, lag -1; $P$ <0.0001).

All correlations and cross-correlations presented in this study had differential values when culling status was compared. This exploratory study provides information to understand which variables are first affected in case of impaired health resulting in culling, and which variables can be used as predictors in forecasting models. Our findings suggest that deviations in behavioral and production variables along with cross-correlations could be observed in animals with higher culling risk.

*Study 2*

Total milk yield from the previous lactation in the cows was 9,578.1 ± 1,696.4 kg (mean ± standard deviation). Therefore, cows whose milk yield exceeded 1,1274 kg were classified as high producing cows (**HP**; n = 16), while cows with previous lactation milk yield less than 7882 kg were classified as low producing cows (**LP**; n = 14). There was no association between lactation number and the milk category ($P$ = 0.55). It was important to evaluate whether the assignment to the production category was related to the individual performance of each cows and not to the lactation number. This is because the objective of this study is to attain locomotion behavior observed in the current lactation to the productive performance of the prior lactation and not to the inherent lactation number.

Locomotion behavior and lameness have an impact in other behavioral variables, milk yield and culling (Randall et al., 2016; Weigele et al., 2017). Lying duration and locomotion activity has been previously analyzed in normal and moderately lame dairy cows, showing that lame cows tend to spend more time lying together with less lying bouts and locomotion activity (Weigele et al., 2017). Additionally, researchers have determined that lame cows produce 1.6 kg/d per milking (King et al., 2017).

In this study, we evaluated the association between locomotion behavior and the milk yield performance in the prior lactation and then to the milk yield in the current lactation. The number of steps per day did not differ between the milk yield category (HP: 1816.46 ± 101.65 vs. LP: 1701.84 ± 102.28; $P = 0.42$). On the other hand, the variable DIM was associated to the number of steps per day ($P < 0.0001$) and it interacted with the milk category ($P = 0.03$). Figure 31 shows the fluctuation in the number of steps per production category throughout the monitoring period (7 d pre-calving and 50 DIM).

No differences were observed in lying bouts between HP and LP cows (8.5 ± 0.5 vs. 9.03 ± 0.6 n/d; $P = 0.5$). Unlike steps per day, DIM was significantly associated to the number of lying bouts ($P < 0.0001$) and it interacted with the milk category ($P = 0.03$). Lying bouts behavior throughout the monitoring period is presented in Figure 32.

**Table 12.** Pre- and post-partum **s**imple linear correlation and cross-correlation estimates of behavioral parameters (active, ruminating and eating time) and milk yield in study cows (N = 2) and in culled cows before 60 DIM (cases) and healthy control cows. Time lags of the greatest cross-correlations and *P*-values are included. NS: non-significant cross-correlation.

Pre-partum

| | Cases (n = 12) | | | | | Controls (n = 30) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Varibles | Simple Correlation | *P* -value | Cross-correlation | Lag | *P* -value | Simple Correlation | *P* -value | Cross-correlation | Lag | *P* -value |
| Rumination / Activity | 0.48 | 0.04 | 0.51 | 1 | 0.03 | -0.15 | 0.52 | NS | | |
| Rumination / Eating time | -0.32 | 0.18 | 0.49 | -6 | 0.04 | -0.98 | <0.0001 | -0.53 | 1 | 0.028 |
| Activity / Eating time | -0.63 | <0.01 | -0.63 | 2 | <0.01 | 0 | 0.97 | NS | | |

Post-partum

| | Cases (n = 12) | | | | | Controls (n = 30) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Varibles | Simple Correlation | *P* -value | Cross-correlation | Lag | *P* -value | Simple Correlation | *P* -value | Cross-correlation | Lag | *P* -value |
| Rumination / Activity | -0.1 | 0.53 | 0.33 | 6 | 0.03 | -0.77 | <0.0001 | -0.63 | -1 | <0.0001 |
| Rumination / Eating time | 0.61 | <0.0001 | -0.62 | 7 | <0.0001 | 0.31 | 0.04 | 0.62 | -3 | <0.0001 |
| Activity / Eating time | 0.12 | 0.76 | NS | | | -0.75 | <0.0001 | -0.63 | 1 | <0.0001 |

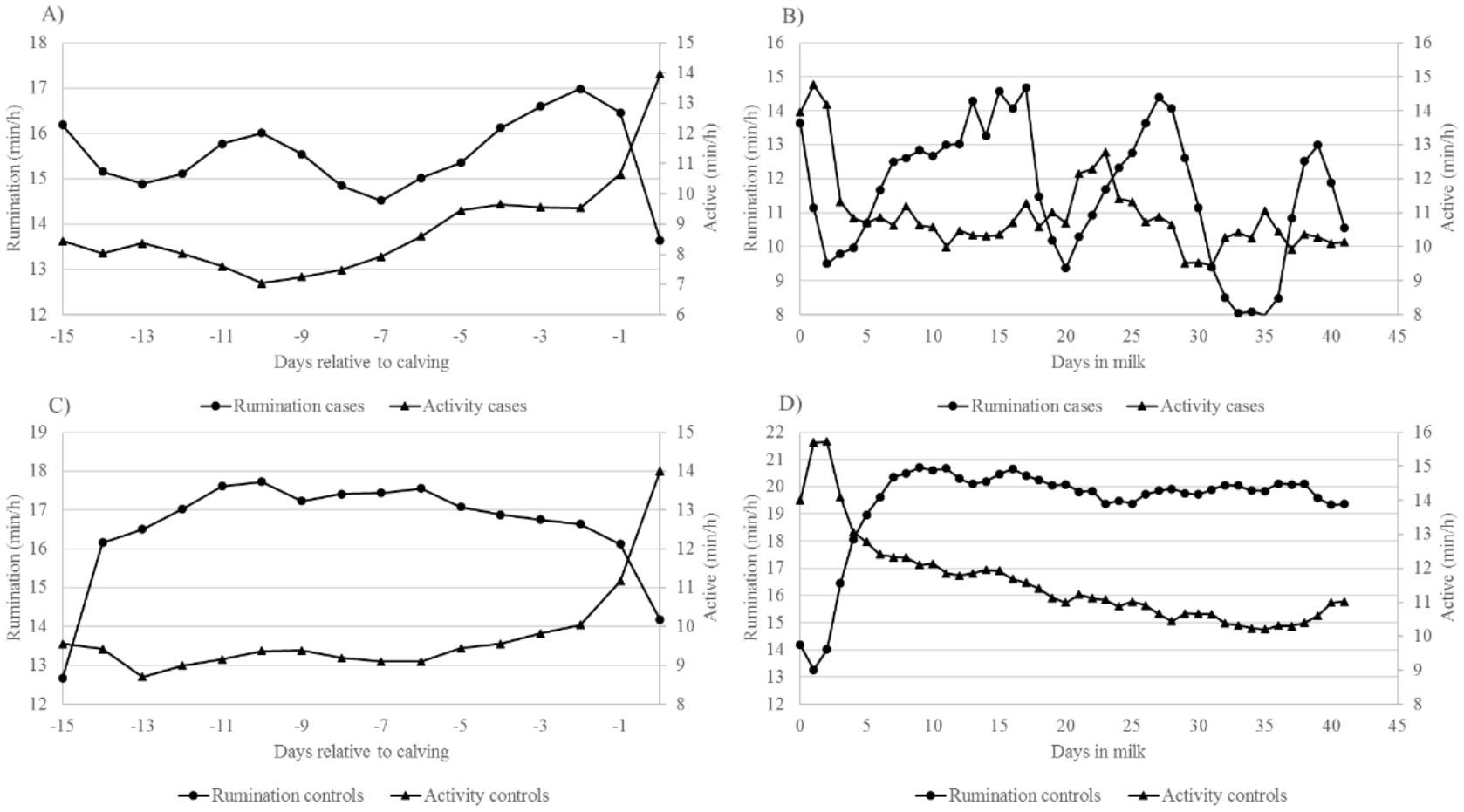



**Figure 28.** Pre- and post-partum cross series plots between rumination and activity (min/h) by culling status. A) Cases' pre-calving cross series plot, simple correlation: 0.48; $P = 0.04$, cross-correlation: 0.51; lag: 1, $P = 0.03$. B) Cases' post-calving cross series plot, simple correlation: -0.1, $P = 0.53$, cross-correlation: 0.33, lag 6, $P = 0.03$. C) Controls' pre-calving cross series plot, simple correlation: -0.15, $P = 0.52$, cross-correlation: non-significant (NS). D) Controls' post-calving cross series plot, simple correlation: -0.77, $P < 0.0001$, cross-correlation: -0.63, lag -1, $P < 0.0001$

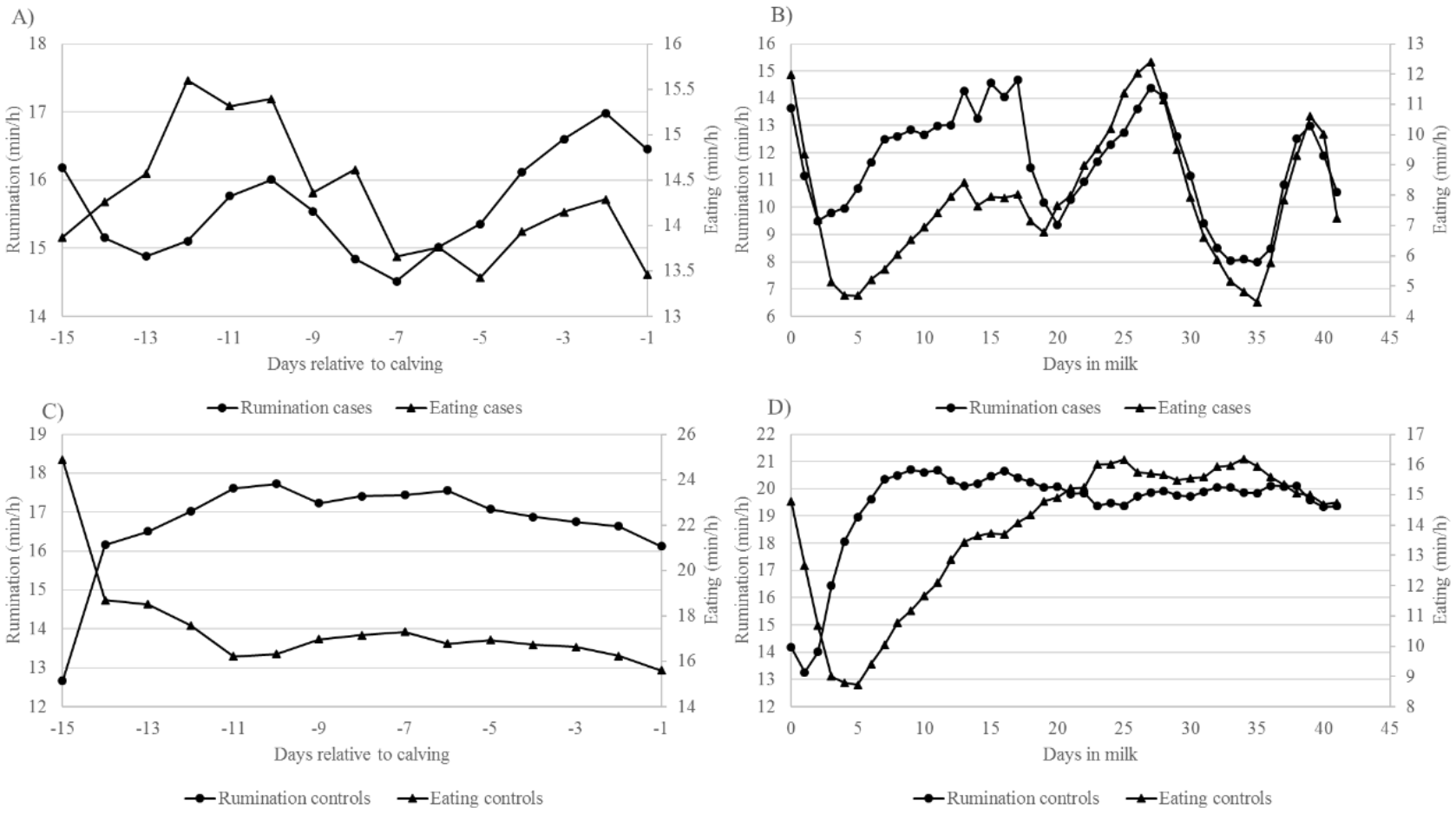



**Figure 29.** Pre- and post-partum cross series plots between rumination (min/h) and eating time (min/h) by culling status. A) Cases' pre-calving cross series plot, simple correlation: -0.32; $P = 0.18$, cross-correlation: 0.49; lag: -6, $P = 0.04$. B) Cases' post-calving cross series plot, simple correlation: 0.61, $P < 0.0001$, cross-correlation: -0.62, lag 7, $P < 0.0001$. C) Controls' pre-calving cross series plot, simple correlation: -0.98, $P < 0.0001$, cross-correlation: -0.53, lag1, $P = 0.028$. D) Controls' post-calving cross series plot, simple correlation: 0.31, $P = 0.04$, cross-correlation: 0.62, lag -3, $P < 0.0001$

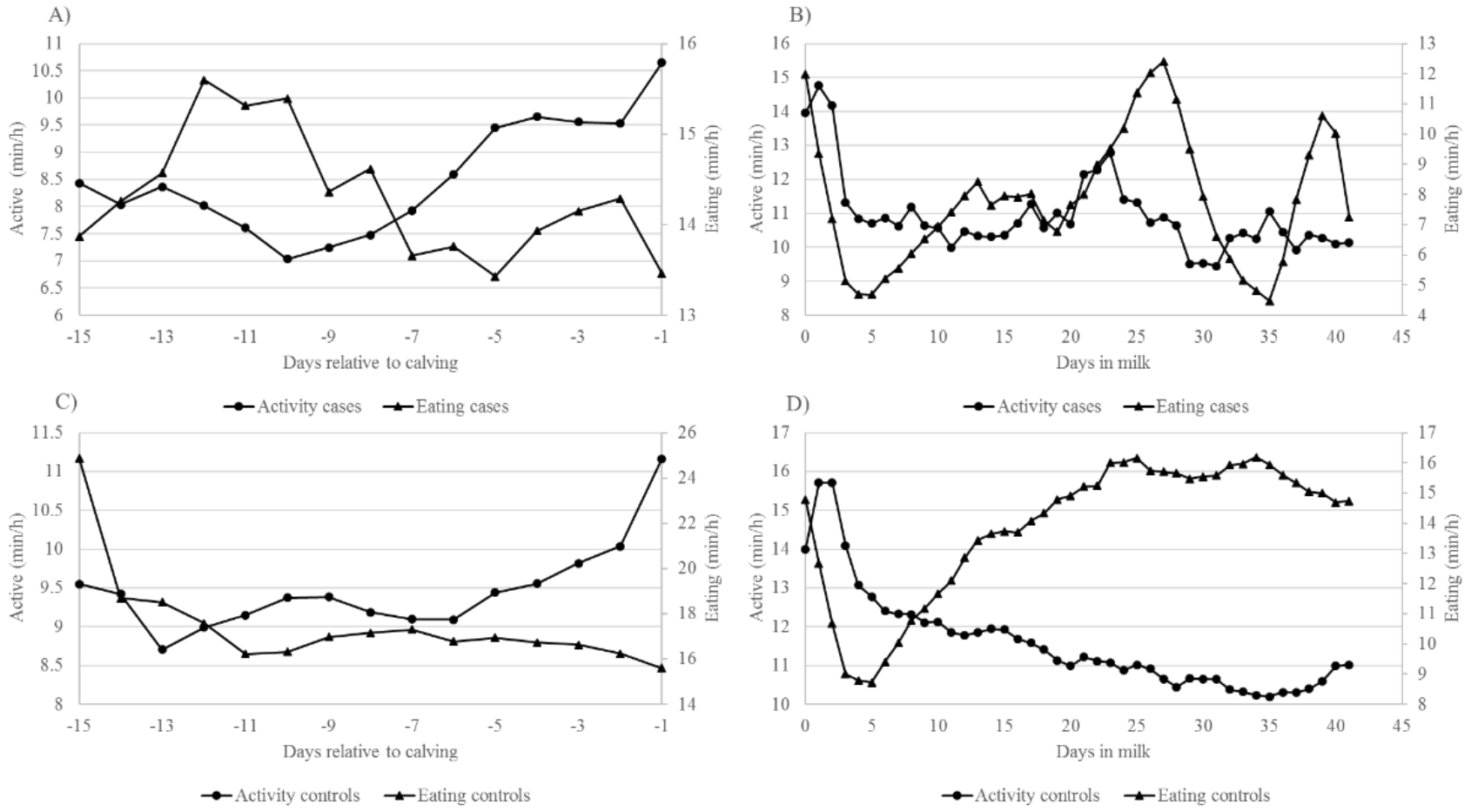



**Figure 30.** Pre- and post-partum cross series plots between activity and eating time (min/h) by status. A) Cases' pre-calving cross series plot, simple correlation: -0.63; $P < 0.01$, cross-correlation: -0.63; lag: 2, $P < 0.01$. B) Cases' post-calving cross series plot, simple correlation: 0.12, $P = 0.76$, cross-correlation: non-significant (NS). C) Controls' pre-calving cross series plot, simple correlation: 0, $P = 0.97$, cross-correlation: non-significant (NS). D) Controls' post-calving cross series plot, simple correlation: -0.75, $P < 0.0001$, cross-correlation: -0.63, lag 1, $P < 0.0001$.

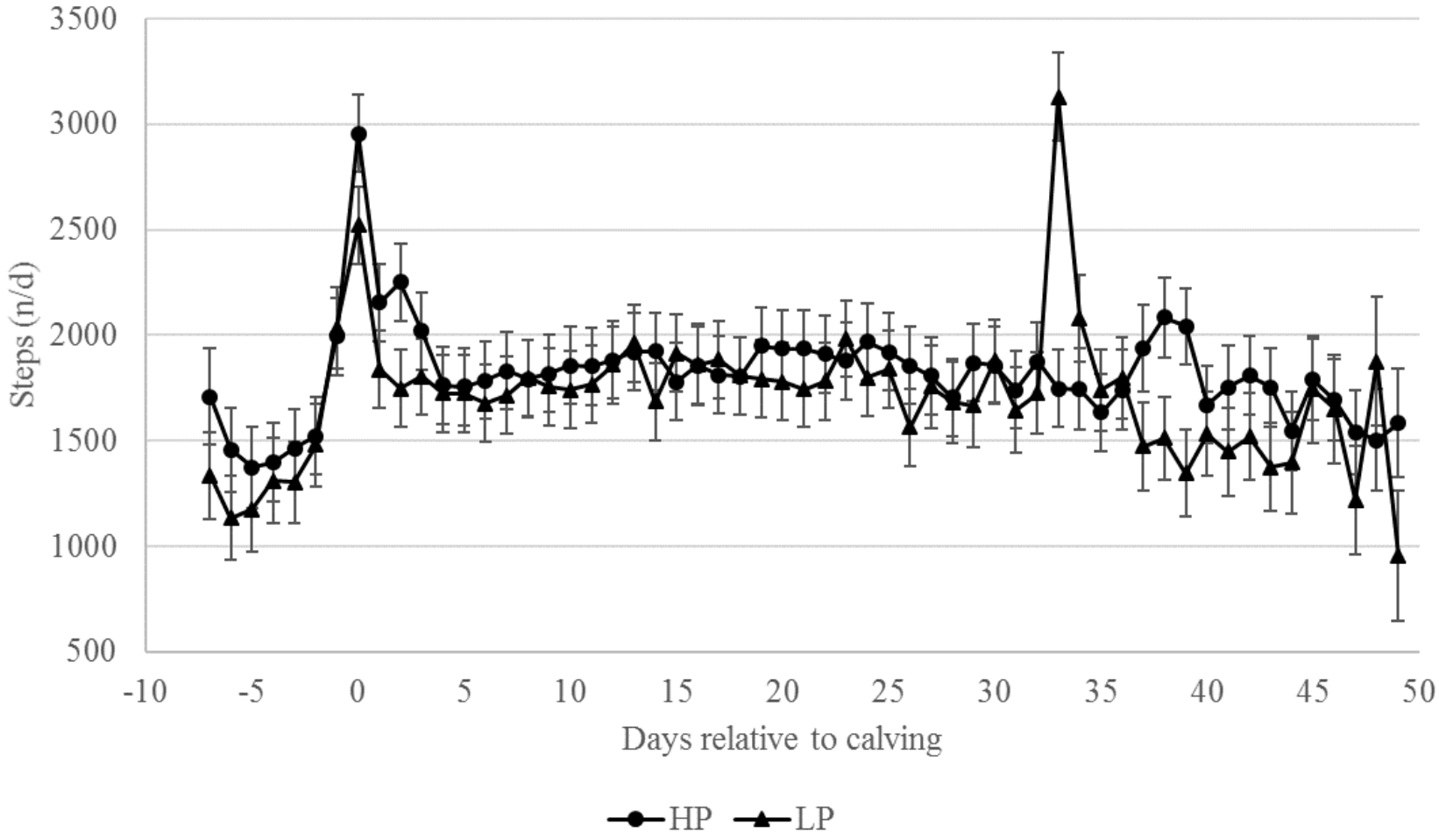



**Figure 31.** Number of steps during 7 days pre-partum until 50 DIM in high producing (HP) and low producing dairy cows (LP) classified based on prior lactation milk yield. Day 0 represents the calving day

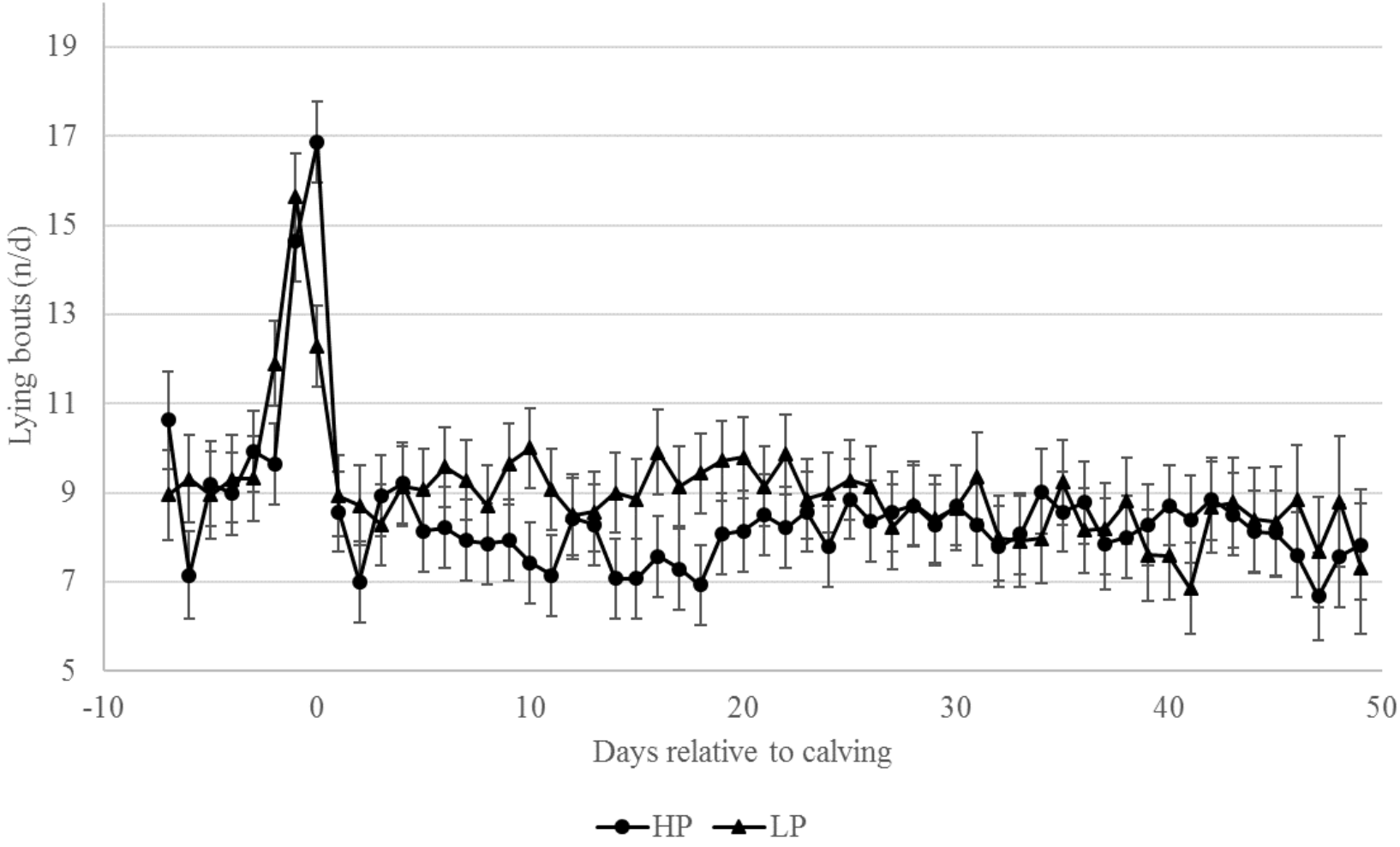



**Figure 32.** Number of lying bouts during 7 days pre-partum until 50 DIM in high producing (HP) and low producing dairy cows (LP) classified based on prior lactation milk yield. Day 0 represents the calving day.

The analysis of lying bouts should consider additional information since a greater number might not represent the time that cows rest or ruminate. Therefore, we compared the time (h/d) that cows from both production categories spent lying. Cows categorized as HP had greater lying time per day compared to LP cows (10:06 ± 00:15 vs 09:14 ± 00:15 hh:mm; $P = 0.01$). A significant interaction between production category and DIM ($P = 0.003$) showed differential patterns of lying time between the study groups especially during the first 21 DIM (Figure 33). Conversely, Fregonesi and Leaver (2001) determined that HP dairy cows had lower lying time compared to LP cows. Nonetheless, lying behavior was assessed weekly by a period of 24 h by a team of observers, which might be subject to human errors and may not reflect the continuous behavior of lactating dairy cows.

The analysis of rumination resulted in similar ruminating behavior between HP and LP cows from pre-calving up to 50 DIM (20.06 ± 0.79 vs. 20.83 ± 0.9 min/h; $P = 0.51$). Additionally, there was no interaction between production category and DIM ($P = 0.97$).

The main differences between the production categories was lying time. As depicted in Figure 33, the greatest deviations were observed after calving. Hence, we hypothesized that those differences could be associated with milk yield. In order to study the association of lying time and daily milk yield up to 50 DIM, we investigated whether greater lying time was associated with daily milk yield in the current lactation. Consequently, a linear regression model was built. This model had milk yield as response and lying time, production category, and lactation number. Production category was not associated to milk yield at 50 DIM ($P = 0.96$) and it was subsequently removed from the model.

Thus, the final linear regression model considered average lying time at 21 DIM ($P$ = 0.003) and lactation number ($P$ = 0.02). Estimates obtained from the linear regression are presented in Table 13. A positive association was found between lying time and milk production where increasing lying time average also increases milk yield.

**Conclusions**

We observed significant associations between active time, ruminating and eating time and culling due to health reasons before 60 DIM. Culled animals had marked lower levels in behavioral variables right after calving. Cross-correlation analysis suggested differential potential in bivariate temporal relationships by culling status during pre- and post-partum regarding the magnitude, direction and time lags. This information could lead future research in autoregressive models for disease and culling with information collected during peripartum. There were no associations between serum concentrations of glucose, BHB, NEFA and insulin and culling. Further research is needed increasing the sample size, as well as considering specific health disorders.

Productive performance in prior lactations is associated to the lying behavior in the subsequent lactation. Thereby, HP cows have significantly higher lying time compared to LP cows, especially during the first 21 DIM. Additionally, increments in lying time are positively associated with increase in daily milk yield in the current lactation.

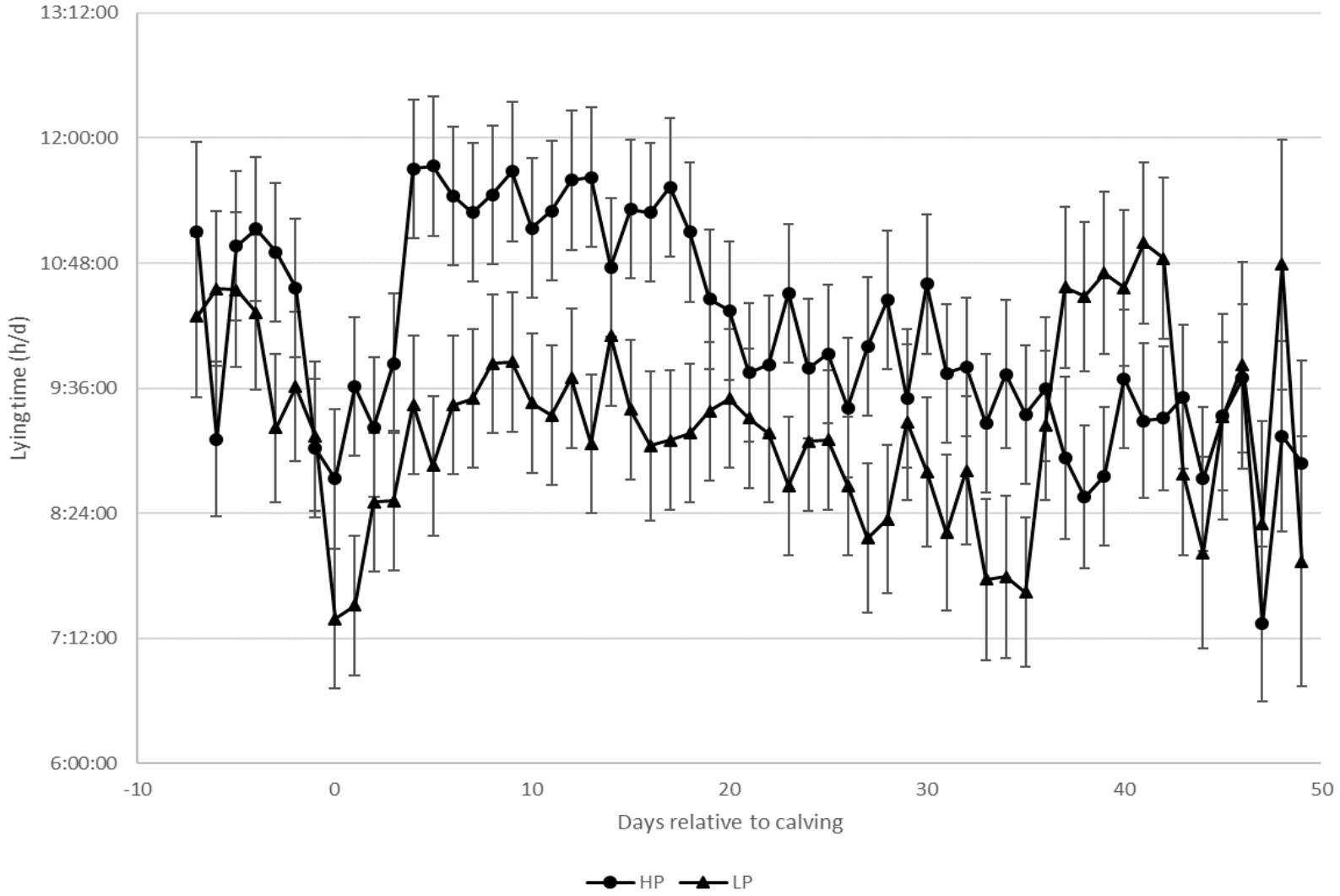



**Figure 33.** Daily lying time during 7 days pre-partum until 50 DIM in high producing (HP) and low producing dairy cows (LP) classified based on prior lactation milk yield. Day 0 represents the calving day.

**Table 13.** Parameter estimates of significant variables associated with daily milk yield until 50 DIM in a linear regression model.

| Variable | Estimate | SE | *P* -value | R-Square |
|---|---|---|---|---|
| Model | | | 0.002 | 0.54 |
| Intercept | 4.31 | 9.25 | 0.64 | |
| Lying time (h/d) | 74.07 | 22.86 | 0.003 | |
| Lactation number | | | 0.02 | |
| 2 | -0.47 | 1.64 | 0.7 | |
| 3 | -0.86 | 1.74 | 0.6 | |
| 4 | 6.28 | 1.77 | 0.002 | |
| 5 | -2.38 | 3.04 | 0.44 | |
| 6 | - | - | - | |

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# CHAPTER 4 - DEVELOPING OBJECTIVE MEASURES OF WELFARE FOR ORGANIC HERDS BASED ON BEHAVIORAL DATA, CYCLICITY, PRODUCTIVE PERFORMANCE AND CLINICAL DISEASE

## Introduction

Scientists and dairy farmers are concerned about the raising mortality and culling rates, especially in intensive production systems (De Vries et al. 2010; Compton 2017). Genetic selection for increased milk yield is usually accompanied by lower fertility, increasing incidence of health problems, and reduced longevity, which are welfare issues in the modern dairy industry (Oltenacu and Algers, 2005). Since culling and mortality have an impact in farm profitability and animal welfare (Compton et al., 2017), culling and mortality rates have been pointed out as poor welfare status indicators within dairy farms (De Vries et al., 2010). For these reasons, the study of factors associated with culling and mortality has gained interest among researchers, especially in animal-based assessments (Fregonesi and Leaver, 2001; De Vries et al., 2010). In general terms, the evaluation of the number of culled and dead animals (incidence risk and density), disease occurrence, injuries, and locomotion's scores are recognized as animal-based indicators of welfare in dairy cows (Capdeville and Veissier, 2001; Fregonesi and Leaver, 2001; De Vries et al., 2010; Whay and Shearer, 2017). Most of these factors plus management, facilities and environmental conditions are permanently assessed in welfare audits in US farms.

Impaired welfare in dairy animals is evaluated by conditions or events that affect the five freedoms that provide the bases of good animal welfare in a productive context. These freedoms include: freedom of hunger and thirst, freedom from discomfort, freedom from pain, injury and disease, freedom to express normal behavior and freedom from fear and distress (Whay and Shearer, 2017). Although standardizations in the methodologies evaluating animal welfare at farm

level provide valuable information about critical control points, these evaluations rely on observers' expertise and their consistency to evaluate variables from animals, workers, and facilities. Moreover, welfare audits are characterized by cross-sectional evaluations that might not ensure the coverage of welfare criteria in a daily basis. In this sense, remote sensor devices (**RSD**) provide an opportunity for continuous evaluation of animal behavior as indicator of welfare (Haley et al., 2000; Borchers et al., 2016) if differential patterns of behavior are associated to achievements of productive goals, without impaired health and/or reproduction causing animal suffering or unnecessary stress.

*Study hypothesis and general objective*

We hypothesized that an appropriate level of welfare is reflected by absence of clinical disease, efficient fertility, and adequate milk production levels and differential patterns of activity, rumination and eating time that could be used to establish real-time behavioral baselines for welfare assessment in dairy farms. Therefore, the objective of this study was to investigate associations between behavioral parameters and welfare status defined as by absence of clinical disease and efficient reproduction and productivity performance.

**Materials and Methods**

*Study animals and management*

The Institutional Animal Care and Use Committee of the Colorado State University (Protocol ID: 16-6704AA) reviewed and approved all procedures performed on the study cows. A single cohort of two-hundred and two (Multiparous [**MP**] = 147; Primiparous [**PP**] n = 55) Holstein cows were followed from 10 d pre-calving until 150 DIM from January 12$^{th}$ to July 7$^{th}$, 2016. At enrollment, 10 d prior to the expected calving date, all cows were ear-tagged with a RSD that recorded activity (min/h), rumination (min/h), and eating time (min/h). All study cows shared the

same management conditions at dry-off, housing in maternity, pre-, and post-calving procedures. After calving, the enrolled animals were assigned to the same research fresh pen used for the study purposes only. After calving, all study animals had the same milking schedules (3 per day), health checks, reproduction management, and feeding diets and times. At 80 ± 11 DIM, study cows started grazing season, in which at least 30% of the dry matter intake was provided from pasture.

*Classification of welfare status*

The welfare status of the study cows was determined by retrospective analysis of cow health and performance. After 150 d of monitoring since calving date, cows with absence of clinical disease (including digestive, respiratory, reproductive disorders, lameness and toxic mastitis), resumed ovarian cyclicity at 50 DIM, milk yield performance equal or above the study group mean, and still at the farm by 150 DIM were classified within the welfare category 1 (**WC-1**) constituted by animals without clinical disease and in good reproductive and productive performance. On the other hand, cows that did not accomplish any of the above conditions were classified within the welfare category 2 (**WC-2**)

Additionally, we separately assessed the effect of parity on the welfare category proposed in this study. In PP cows, the welfare category did not include the cycling outcome; hence, WC-1 were defined by absence of clinical disease, culling or mortality, and milk yield on or above the average among PP cows. However, since milking groups in dairy farms contain PP and MP cows, we present the results using the whole data set and then stratified by parity.

*Behavioral, productive, health and reproduction outcomes*

An ear-tag accelerometer (CowManager SensOor, Agis Automatisering BV, Harmelen, the Netherlands) continuously (min/h) recorded active, rumination and eating time (min/d) during the observation period. Raw data from each study cow was received daily and a data set containing cow ID, welfare group, parity and calving date was generated for the analysis.

Trained farm personnel performed daily health checks. Complete clinical examination was performed during the first 21 DIM on each study cow. Briefly, health checks consisted in assessment of retained placenta determined by retained fetal membranes for more than 24 h. Metritis determined by fetid vaginal discharge and abnormal fluids in the uterus at trans-rectal palpation. Ketosis was determined using urine ketosis strips in animals with systemic symptoms such as watery manure, foul smelling in breath and depression, Digestive problems such as bloating, bloody manure, constipation and scours determined by auscultation, percussion and manure examination.

Finally, respiratory system was evaluated by presence of nasal discharge, cough and respiratory rate. Additionally, rectal temperature and animal attitude was part of the health checks. Data from health disorders events, treatments, and movements to the hospital group were obtained from farm records.

Resumption of ovarian cyclicity was the main reproductive outcome included in this study. Our research team performed transrectal ultrasound of the reproductive tract at 35 ± 3 DIM and at 49 ± 3 DIM. The presence of one corpus luteum (CL) determined resumption of ovarian cyclicity. Cows having a CL at the 35 DIM evaluation were not further assessed. Resumption of ovarian cyclicity was recorded as binary variable (1 = CL presence at 35 or 49 DIM; 0 = no CL at both evaluations).

*Statistical analysis*

Differences in activity, rumination and eating time among the animals classified as WC-1 and WC-2 were calculated using PROC GLIMMIX in SAS (SAS 9.4, SAS institute Inc., Cary, NC) for the observation period. The model examining the whole data set included welfare category (for MP cows), DIM, and the interaction term between welfare category and DIM. In the stratified analysis using the different welfare criteria for MP and PP cows, the models included the effect of the welfare category and DIM as well as their interaction term.

To investigate thresholds of behavioral parameters associated with the likelihood of being categorized as WC-1 or WC-2, PROC LOGISTIC was used considering welfare category as binary response (WC-1 = 1; WC-2 = 0), and accumulated time of behavioral variables as explanatory variables. For significant associations, odds ratios were calculated using 5 minutes increase of behavioral variables.

Receiving operanting curves (ROC) were calculated for each significantly associated behavioral variable to determine cut-off values that could define welfare status in a specific period of the lactation. Statistical significance was determined at *P*-value <0.05 and tendency at *P*-value ≤0.15.

**Results and Discussion**

The study of animal behavior offers valuable possibilities to detect health disorders due to the relationship between behavioral responses and the animal internal state and the environment (Weary et al., 2009). The definition of animal welfare in lactating dairy cows must be applied in a production system context, where all management practices leading to the desired production outcomes also consider the five freedoms, especially the absence of painful or life-threatening diseases, and the optimal expression of normal behavior. In this study, we evaluated behavioral

deviations among animals classified by their health, productive and reproductive performance. Overall, fifty-nine (29.5%) cows were classified as WC-1 whereas 141 (70.5%) as WC-2. The stratification by parity resulted in 56 (38.1%) MP cows classified as WC-1 and 91 (61.9%) as WC-2. On the other hand, the monitoring of PP cows resulted in 26 (47.3%) cows classified as WC-1 and 29 (52.7%) as WC-2.

When both MP and PP cows were included in the analysis, active time did not differ between animals classified as WC-1 and WC-2 (11.7 ± 0.24 vs. 11.55 ± 0.15 min/h; $P = 0.48$). The covariate DIM and the interaction term between the welfare performance category and DIM resulted significant ($P < 0.0001$). Before the analysis, we did not expect a substantial difference in the time that the study cows remained active because all pen movements and space available were share by all cows. Active time fluctuations are graphically presented in Figure 34.

Among the study animals, the association of welfare category with rumination showed a trend ($P = 0.08$). On average during the observation period, WC-1 cows ruminate 20 ± 0.4 min/h, while WC-2 ruminate 19.24 ± 0.23 min/h.

Days in milk had a significant association with rumination time as well as the interaction time between DIM and the welfare status covariate ($P < 0.0001$).

The fluctuations in rumination observed throughout the monitoring period are shown in Figure 35. The significance of the interaction term makes important the study of the deviations at different stages on the lactation, where differential behavior could be used for welfare assessment. As observed in Figure 35, there were no clear pre-partum differences among cows that will be categorized in the WC-1 or WC-2 categories by the end of the observation period. Contrastingly, sound deviations in rumination were observed during the first 21 DIM, and after 90 DIM, which is a period where study cows were grazing. Regarding rumination differences during grazing, the

development of behavioral baselines associated with a welfare status during this season may significantly improve health management especially in certified organic farms. This is because organic farmers are required to pasture lactating cattle no less than 120 per year and ensure that no less than 30 percent of the dry matter intake comes from grazing (NOP, 2013). Therefore, monitoring rumination in organic dairies can be a useful tool for detecting disease, controlling reproductive status and estimating dry matter intake (Rombach et al., 2018).

Rumination behavior in the study cows had differential patterns during grazing. During grazing, as observed in Figure 35, dairy cows reduced rumination as a compensatory mechanism to increase effective grazing when time at pasture is restricted (Gregorini et al., 2012). Cows that will have a good overall performance up to 150 DIM (WC-1) maintained rumination levels around 20 min/h right after the start of grazing, while cows with impaired performance had a greater drop in rumination after the pasture management started and showed lower time of rumination throughout the grazing management (Figure 35).

The observed differences within the first 21 DIM and after grazing offer a possibility to establish thresholds of rumination that might be associated with good health and productivity standing in lactating cows. Moreover, the continuous assessment of rumination time using RSD could be used for real-time welfare evaluation of lactating groups in the farm.

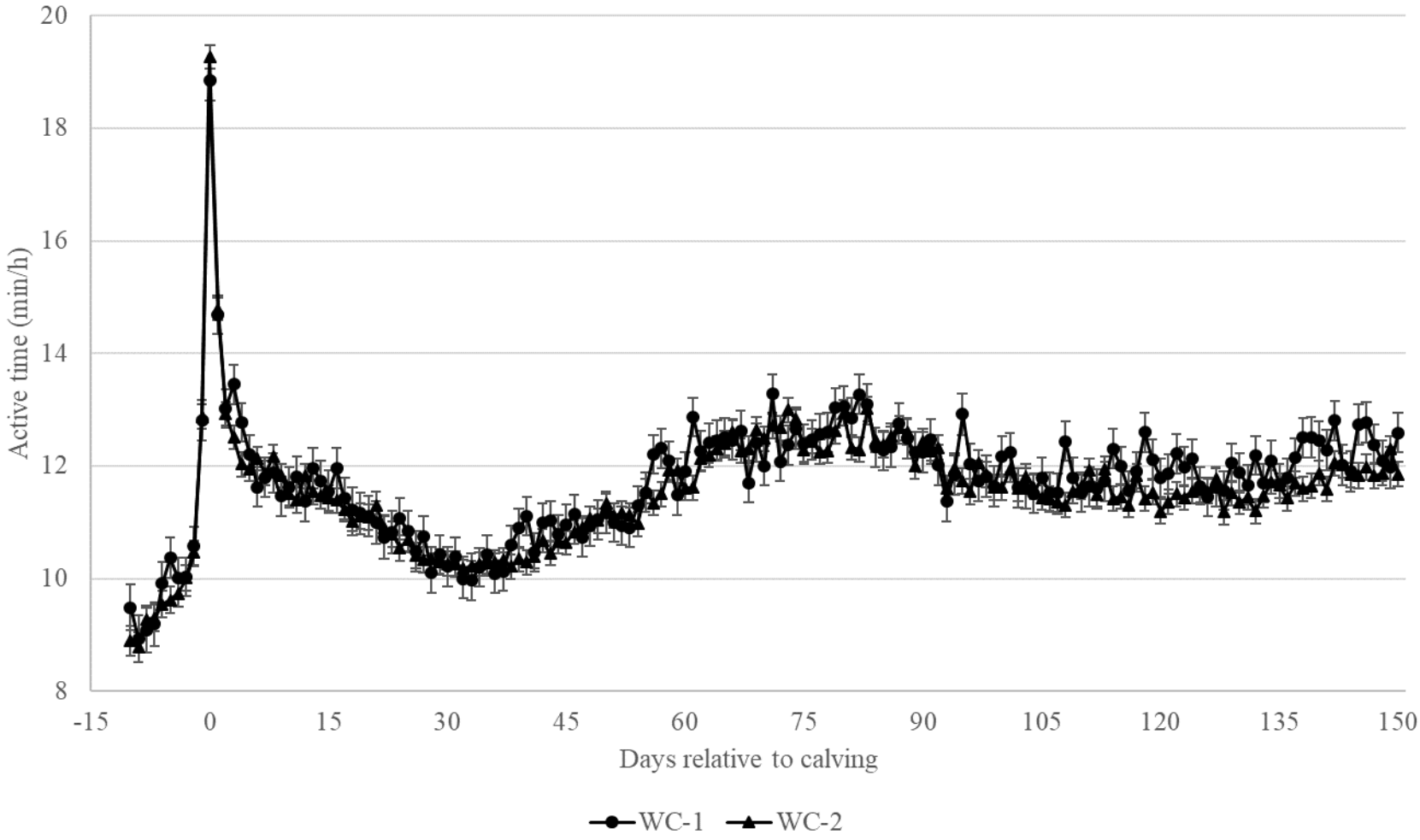


**Figure 34.** Daily active time average (min/h) in multiparous and primiparous dairy cows classified as having good health and productive standing (WC-1, n = 59) and poor health and productive standing (WC-2, n = 141) observed from 10 days prior to calving until 150 DIM. WC-1 (Welfare Category 1): animals without clinical disease and in good reproductive and productive performance until 150 DIM. WC-2 (Welfare Category 2): animals that did not accomplish any of WC-1.

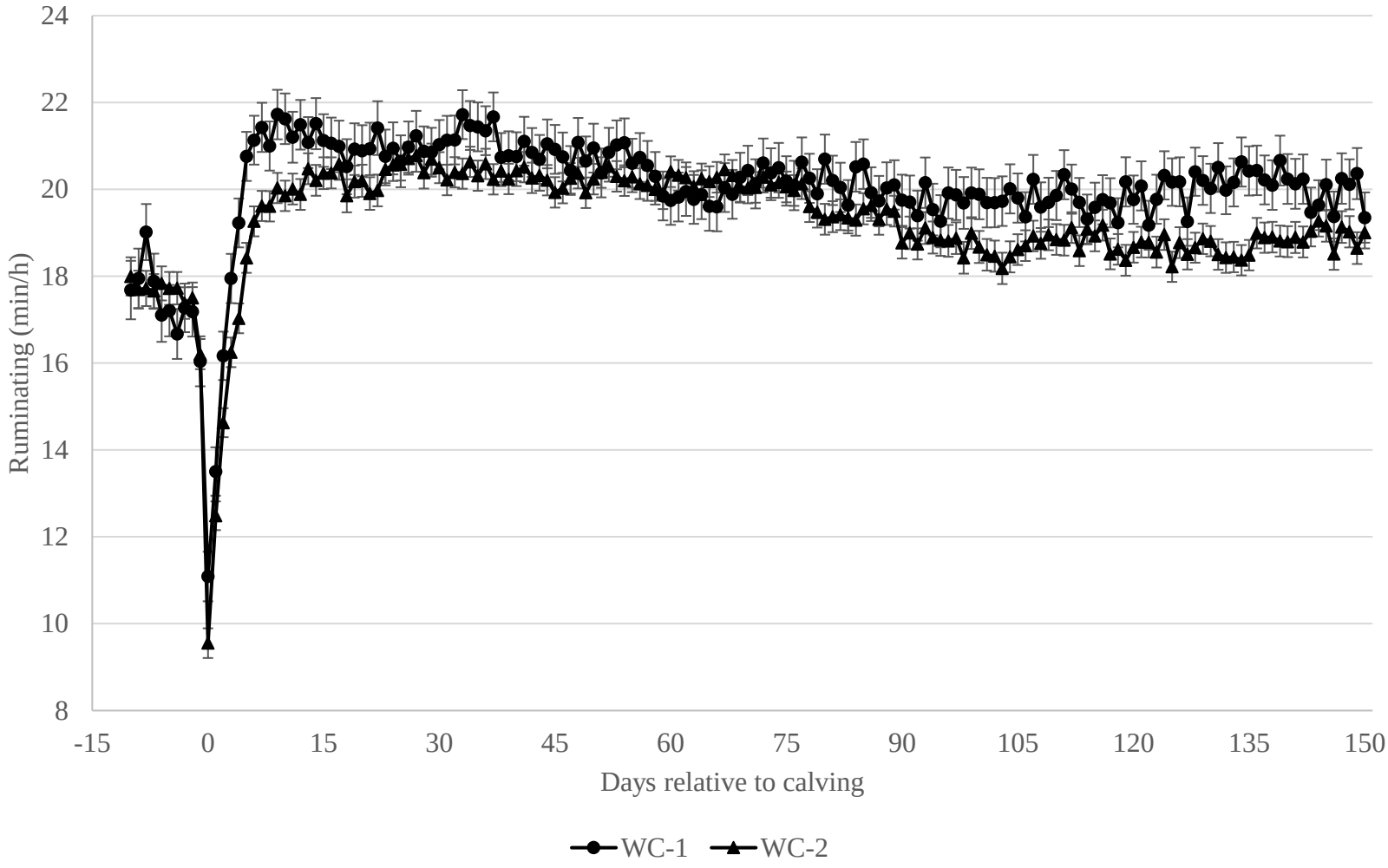


**Figure 35.** Daily rumination average (min/h) in multiparous and primiparous dairy cows classified as having good health and productive standing (WC-1, n = 59) and poor health and productive standing (WC-2, n = 141) observed from 10 days prior to calving until 150 DIM. WC-1 (Welfare Category 1): animals without clinical disease and in good reproductive and productive performance until 150 DIM. WC-2 (Welfare Category 2): animals that did not accomplish any of WC-1.

Huzzey et al. (2007) suggest that dry matter intake is a potential marker to differentiate healthy cows and cows affected by metritis during the first 21 DIM. In agreement, in the study 1 of chapter 3, we observed that cows culled due to health reasons had differential patterns of eating time. Therefore, we would expect to observe some extent of differentiation between welfare categories although this category also includes productive and reproductive performance, which may not be related to stressing or painful symptoms that sick animals present and affect their behavior (Weary et al., 2009). However, overall eating time estimated from all study animals did not differ by welfare status. WC-1 cows spent on average 14.37 ± 0.5 min/h eating, while WC-2 ate 13.7 ± 0.3 min/h during the monitoring period ($P$ = 0.25). Nonetheless, the significant interaction term between DIM and welfare category ($P$ <0.0001) should be reported and further examined through LSM contrast at defined DIM.

As we defined different criteria for welfare categories for MP and PP, associations between welfare status and daily LSM of the behavioral variables were analyzed separately. In MP cows, the main effect of active time did not differ among WC-1 and WC-2 cows (11.7 ± 0.24 vs. 11.4 ± 0.18; $P$ = 0.37). On the other hand, the interaction term between DIM and welfare category was significant ($P$ <0.0001), however, no major deviations were observed in the graphical assessment (Figure 34).

The analysis of rumination in MP cows resulted in a significant interaction term between DIM and welfare category ($P$ = 0.0003; Figure 36). As showed in Figure 35, MP cows categorized as WC-1 had the same differences with WC-2 during the first 21 DIM, where WC-1 cows at 150 DIM have higher rumination time during that period. Even though overlapping standard error bars are observed in the rumination time LSM after 90 DIM (grazing season), the daily rumination means of WC-1 remains above WC-2 cows.

In MP cows, eating time showed a tendency for significant differences between the welfare categories, where WC-1 cows spent more time eating compared to WC-2 during the monitoring period (14.5 ± 0.5 vs. 13.3 ± 0.4; $P = 0.06$). As with the other response variables, the interaction term between DIM and welfare category resulted significant ($P < 0.0001$), therefore, graphical assessment of eating time between WC-1 and WC-2 category over the observation period is needed to draw conclusions. In MP cows, welfare categories had bigger differences in eating time (Figure 4) compared to activity and rumination. These differences were more evident within the first and second days after calving and remained different until the beginning of the grazing season. In a similar way to rumination, MP cows having better health and productive performance at 150 DIM showed greater eating time during the fresh-cow period (21 DIM), hence, this period could be determined to be critical for welfare assessment and accumulated rumination daily rates could represent a welfare indicator.

Although PP and MP cows were contemporaneous and were exposed to the same management and climatic conditions during this study, we decided to analyze their data separately since the welfare criteria applied to MP cows was harder to reach by PP cows. We found that most PP study cows were not able to resume ovarian cyclicity before 50 DIM, therefore, we did not consider this variable in the welfare categorization of PP cows. Delayed resumption of ovarian cyclicity in PP cows may be given by their higher energy requirements for growth, different metabolic status and nutrient partition during their first lactation (Grummer, 1995; Coffey et al., 2006).

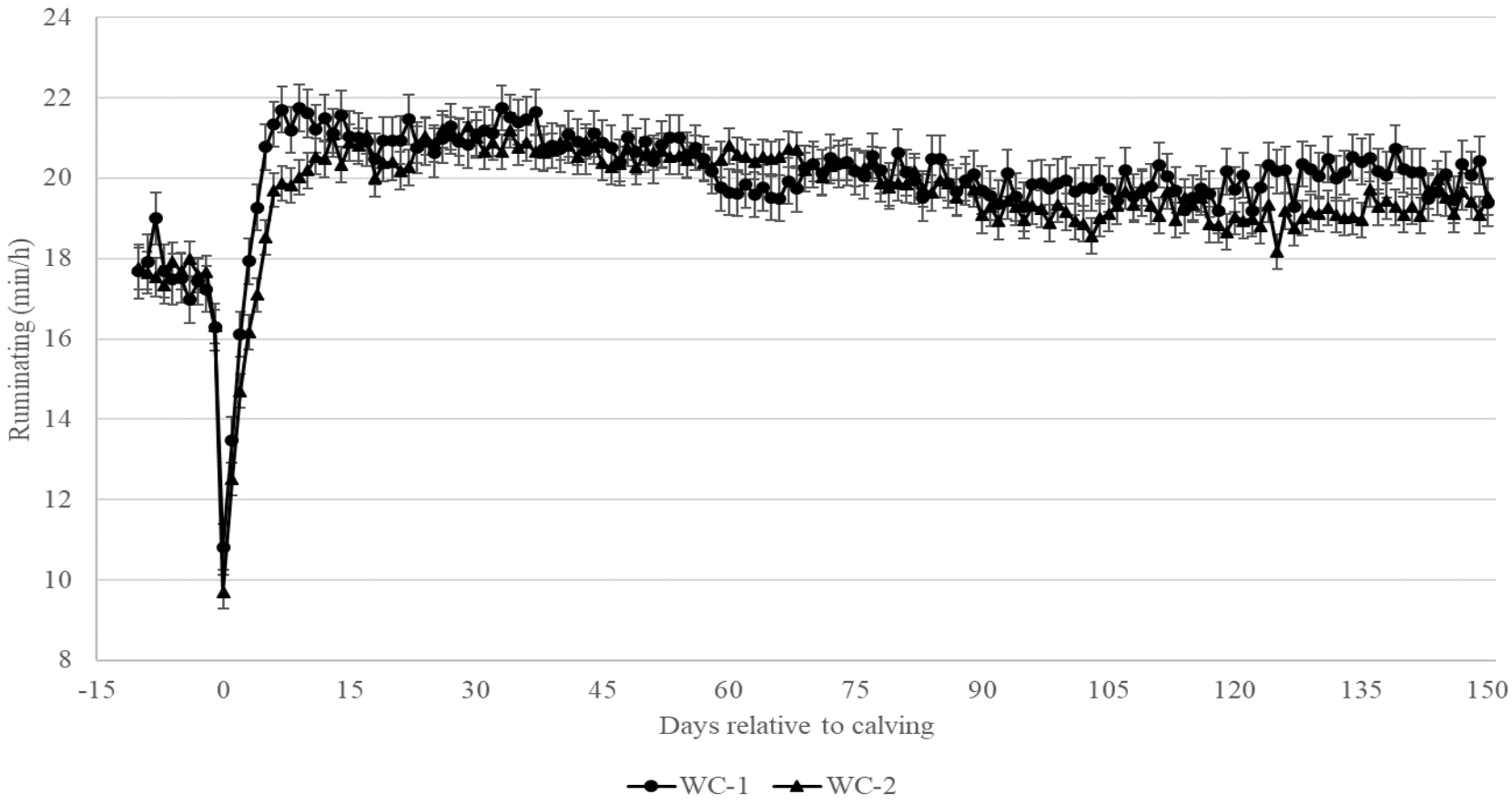


**Figure 36.** Daily rumination time in multiparous dairy cows (n = 147; parity ≥ 2) classified as having good health, reproductive and productive standing (WC-1, n = 56) and poor health, reproductive and productive standing (WC-2, n = 91) observed 10 days prior to calving until 150 DIM. WC-1 (Welfare Category 1): animals without clinical disease and in good reproductive and productive performance until 150 DIM. WC-2 (Welfare Category 2): animals that did not accomplish any of WC-1.

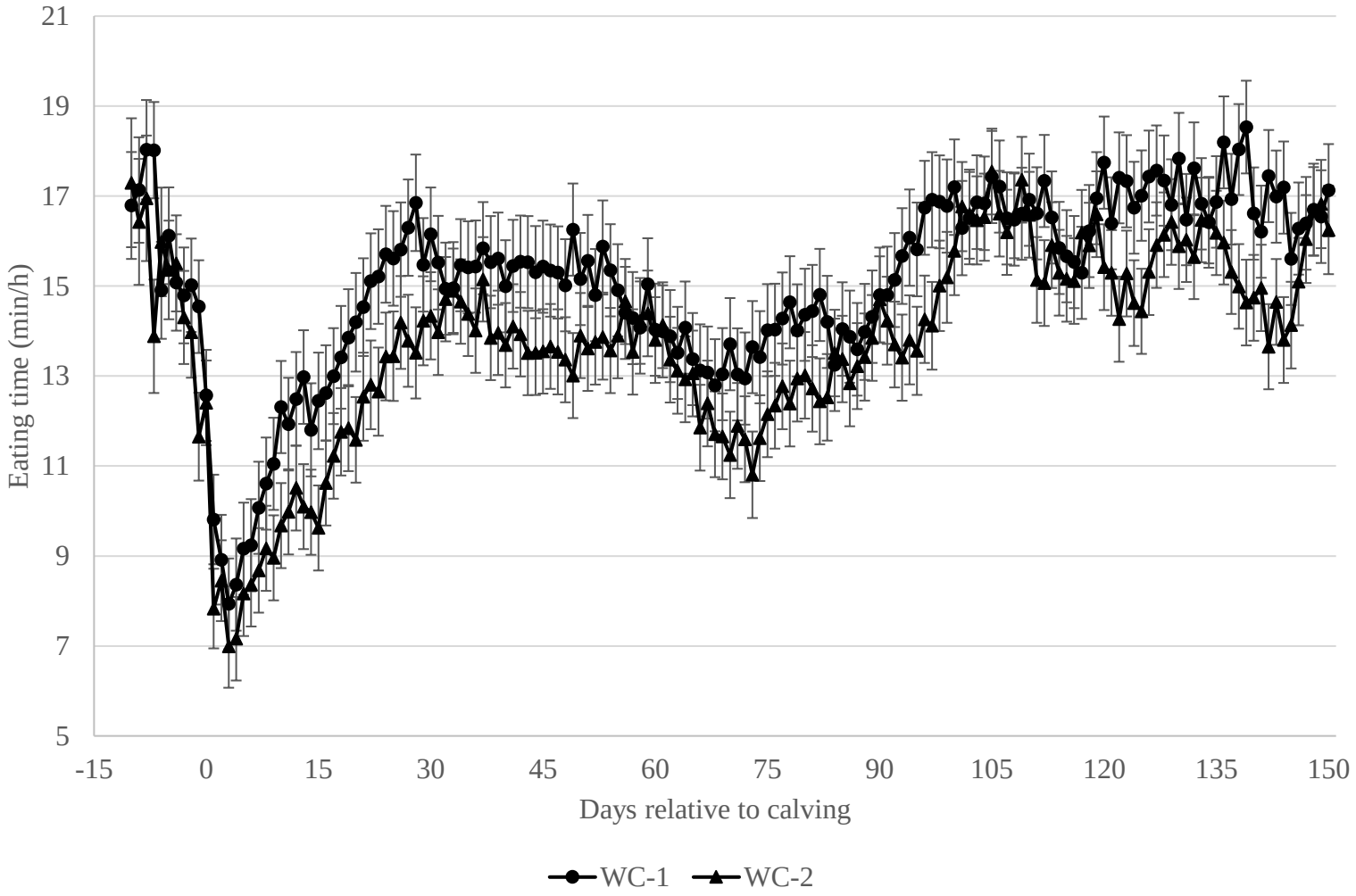


**Figure 37.** Daily eating time average in multiparous dairy cows (n = 147; parity ≥ 2) classified as having good health, reproductive and productive standing (WC-1, n = 56) and poor health, reproductive and productive standing (WC-2, n = 91) observed 10 days prior to calving until 150 DIM. WC-1 (Welfare Category 1): animals without clinical disease and in good reproductive and productive performance until 150 DIM. WC-2 (Welfare Category 2): animals that did not accomplish any of WC-1.

As observed in the analysis using the data without parity stratification, PP cows did not show differences in active time between WC-1 and WC-2 (12.1 ± 0.36 vs. 11.6 ± 0.32; $P = 0.28$). Although the DIM and the interaction term was significant ($P < 0.0001$), the graphical assessment of the activity curves from both welfare groups did not show important deviances throughout the monitoring period.

Rumination has showed to be a very sensitive variable in cases of impaired health and its fluctuation is correlated with changes in productivity (Kaufman et al., 2018). Nonetheless, under the classification of welfare proposed in this study, rumination did not differ among PP cows categorized as WC-1 and WC-2 (19.36 ± 0.58 vs. 18.23 ± 0.52; $P = 0.15$) as main effect. However, it could be considered a tendency in favor of WC-1. The effect of DIM was significant ($P < 0.0001$). However, the interaction term was not significant ($P = 0.98$) meaning that the differences observed at the beginning of the monitoring period between the study group were maintained up to the end of the study (Figure 38). The observed differences were again more substantial within the first 21 DIM.

In PP cows, no differences regarding eating were detected between the observed groups. PP cows classified as WC-1 spent 15.1 ± 0.8 min/h eating, whereas WC-2 cows averaged 13.8 ± 0.7 min/h ($P = 0.22$) eating during the observation time. Again, the model had DIM as significant effect. The interaction term between DIM and the welfare category presented a trend with a *P*-value = 0.08. As observed throughout the study, no differences are observed pre-calving in any of the studied response variables. After calving, the major deviances in eating time are also observed in PP up to 21 DIM and then having similar fluctuations during the lactation (Figure 39).

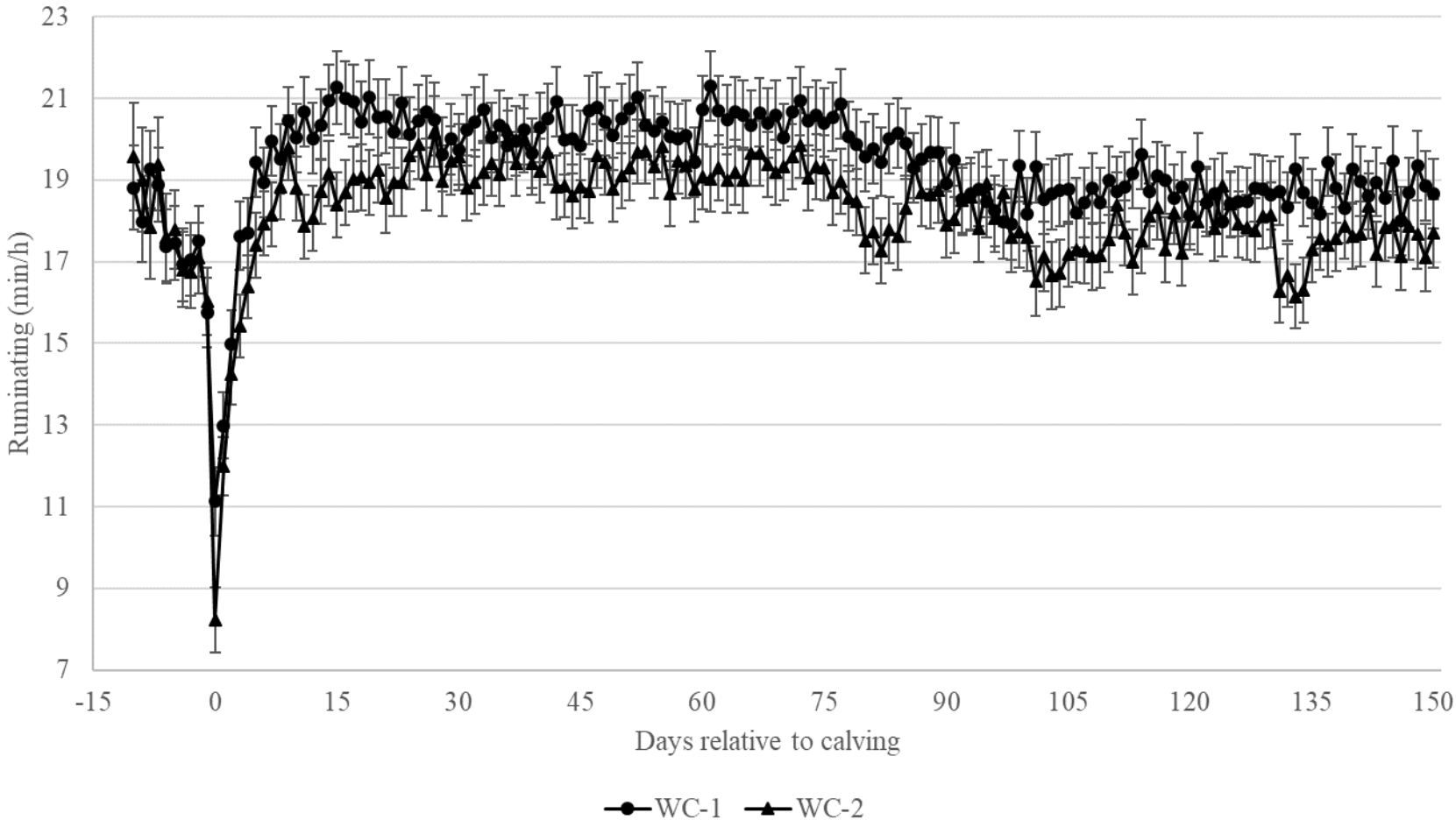


**Figure 38.** Daily rumination time in primiparous dairy cows (N = 55; parity = 1) classified as having good health and productive standing (WC-1, n= 26) and poor health and productive standing (WC-2, n = 29) observed 10 days prior to calving until 150 DIM. WC-1 (Welfare Category 1): animals without clinical disease and in good reproductive and productive performance until 150 DIM. WC-2 (Welfare Category 2): animals that did not accomplish any of WC-1.

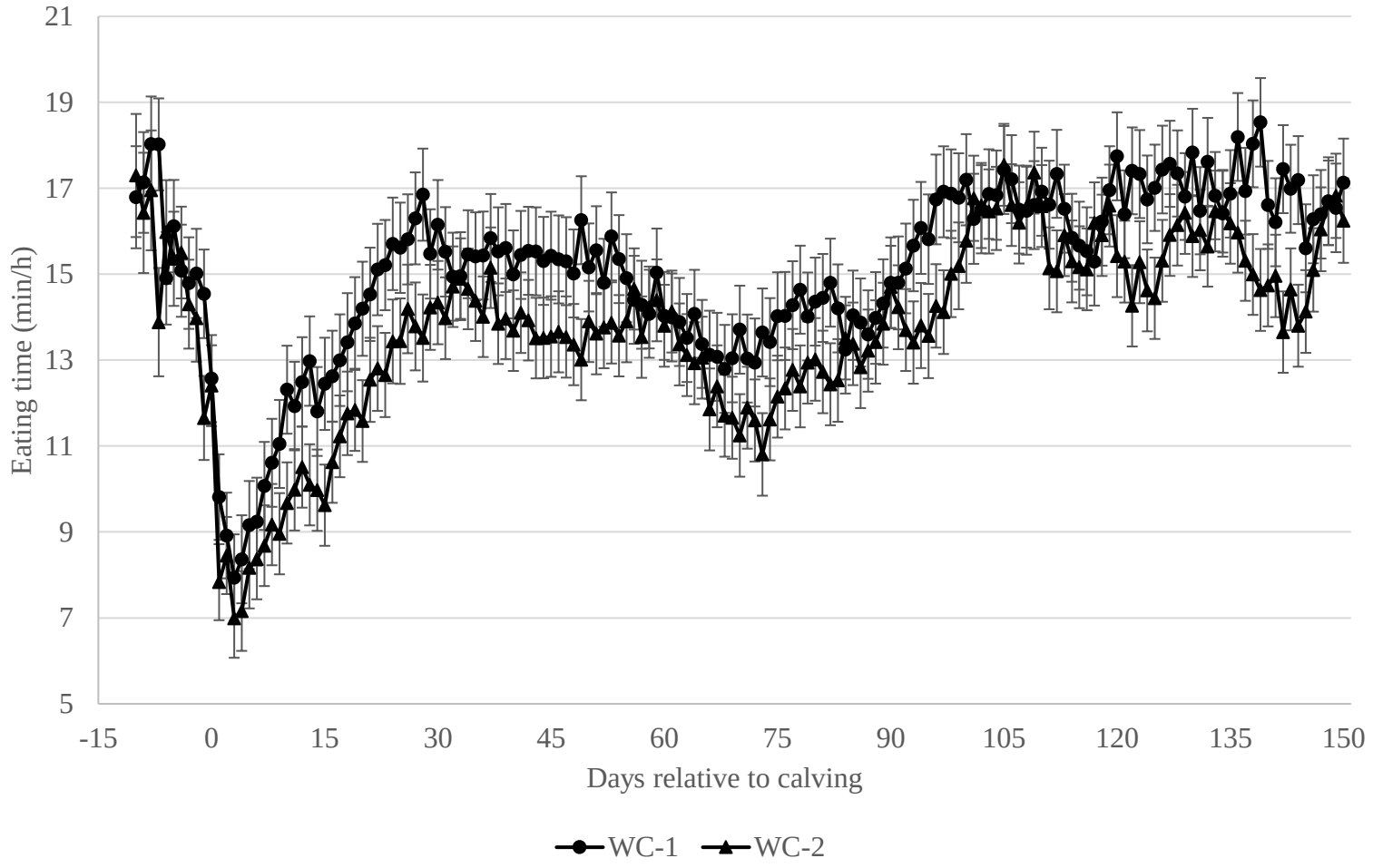


**Figure 39.** Daily eating time in primiparous dairy cows (N = 55; parity = 1) classified as having good health and productive standing (WC-1, n= 26) and poor health and productive standing (WC-2, n = 29) observed 10 days prior to calving until 150 DIM. WC-1 (Welfare Category 1): animals without clinical disease and in good reproductive and productive performance until 150 DIM. WC-2 (Welfare Category 2): animals that did not accomplish any of WC-1.

In this study, rumination and eating time at 21 DIM showed the most significant differences between animals classified as WC-1 and WC-2 at 150 DIM. For this, a different analysis approach used the accumulated average of rumination and eating time were at 21 DIM per study cow as exploratory variable. This was intended to investigate associations between increments of rumination and eating and the likelihood of being classified as WC-1 or WC-2. Additionally, we investigated thresholds of behavioral parameters that could determine welfare status of individual cows during the first 21 DIM. This could be used to assess the welfare status of individual and groups of lactating dairy cows using data being generated daily providing a general view of cow health and behavior as well as to determine which cows are leading to a more successful lactation.

According to De Vries et al. (2010), the daily hazard of culling reaches the highest values within the first 30 DIM regardless of lactation number. Therefore, baselines of rumination and eating time associated to good health and productive standing could contribute to welfare audits and to on-farm assessments. The associations between time (min/h) and the probability of being classified as WC-1 at 150 DIM was calculated for rumination and eating time using all data without parity stratification as well as using differential welfare category criteria for MP and PP cows. Average rumination time calculated at 21 DIM and welfare status had a positive association when the data without considering parity was analyzed ($P$ =0.008). Thus, the odds of being classified as WC-1 increase 2.22 times (95% CI: 1.3 – 4.2) per every 5-min increase in the rumination time average during this period. The investigation of a cut-off in rumination time showed an area under curve (**AUC**) of 0.61. The cut-off value that yielded the highest sensitivity (**Se**) and specificity (**Sp**) was 19.45 min/h (Se = 51%; Sp = 61%).

The association between eating time and welfare category was also positive. Thus, the increment of eating time in 5 units (min/h) increases the odds of classification of WC-1 by 1.6 times (95% CI: 1.1 – 2.45; $P$ = 0.02). Accordingly, the thresholds that provides the highest Se (53%) and Sp (63%) was 11.23 min/h and the AUC was 0.61.

For MP cows, average rumination time during the first 21 DIM was associated with a greater likelihood of being in the WC-1 category. Thereby, increments of rumination in 5 minutes increased the odds of being classified in the WC-1 category by 1.8 times (95% CI: 1.1 – 3.45; $P$ = 0.03). In the search of a cut-off value that could determine whether a MP cow is in a good welfare status that will be translated in an acceptable performance up to 150 DIM, the AUC was 0.56, and the Se and Sp were 51% for the cut-off 19.33 min/h. On the other hand, eating time in MP cows had higher Se and Sp for the association with welfare status. This association indicated that an increase in eating time was associated to greater odds of WC-1 (OR 2.1 [95% CI: 1.3 – 3.4; $P$ = 0.003). Finally, the estimated threshold determining a WC-1 at 150 DIM was 10.7 min/h (Se 65% and Sp 59%).

Primiparous cows had different criteria determining their welfare category. This resulted in a significant association between the increment of rumination and the likelihood of being categorized as WC-1, where 5 min/h increment increased the odds of WC-1 by 3.2 times (95% CI: 1.1 – 11.9; $P$ = 0.05). The predicted probabilities of WC-1 and rumination time are presented in Table 1. The rumination threshold selected in PP cows was 18 min/h yielding a Se of 68% and a Sp of 58%. Conversely to MP cows, in PP cows eating time was not associated to welfare status ($P$ = 0.15), however, it could be considered a statistical tendency (OR presented in Table 14).

The lack of association between increments in eating time and welfare categories in PP cows, may be explained because growing animals show a highly variable eating behavior compared to MP, which is reflected by greater standard errors of the eating time, therefore eating time could be less useful in PP cows to determine welfare status or predict disease.

Several research studies have suggested behavioral parameters measured by RDS as potential indicators to assess overall cow health and to detect disease (Fregonesi et al., 2001; Liboreiro et al, 2015; Borchers et al., 2016, Stangaferro et al., 2016). A better understanding of behavioral fluctuations and specific health and welfare status is needed. Up to date, moderate Se and Sp for methods studying feeding behavior in cows affected by metritis has been reported (Urton et al., 2005). This agrees with our study analysis, where the suggested cut-off values that maximized Se and Sp had moderate Se and Sp values (just above 0.6). However, the associated cut-off values to determine the welfare criteria presented in this study can be modeled depending on the health and productive goals. For example, as the logistic regression odds was modeled towards WC-1, it would be more acceptable to maximize the Sp of the behavioral cut-off, therefore, the number of false positives (misclassified as WC-1) is diminished.

**Conclusions**

According to the welfare criteria applied to the study cows, differential patterns in the rumination and eating time were found between welfare categories. Active time was not associated with the welfare classification. Multiparous cows with better welfare status by 150 had significantly higher average rumination time (min/h) than those showing poor welfare status. In a similar way, successful multiparous cows regarding health, reproductive and productive performance tended to have higher eating time from calving to 150 DIM.

Based on our results, welfare status should be defined separately for multiparous and primiparous cows since some criteria may not apply in the physiology of primiparous cows and should be considered in welfare evaluations. In this study, primiparous cows without clinical disease and with average milk yield by 150 DIM tended to have higher rumination time compared to primiparous cows with impaired health and performance. Higher variability was found in eating time among primiparous study cows and no differences were found between welfare status categories.

Behavioral fluctuations during the first 21 DIM presented the most significant differences between the defined welfare categories. In general, increments in eating and rumination time were associated with increased odds of being categorized in welfare category 1 by 150 DIM. These associations allowed to build significant thresholds of cumulative rumination and eating times that could discriminate our proposed welfare criteria.

Future research directions include evaluation of associations between behavior and welfare at other stages at mid and late lactation

**Table 14.** Summary of associations (odds ratio [OR] and 95% confidence intervals [CI]) between cumulative values for behavior parameters (rumination and eating time), cut-off values (chosen to maximize sensitivity and specificity), sensitivity and specificity, and the area under the curve calculates in simple logistic regression models. Results are presented for analyses performed without (all data) and with parity stratification

| | Variable (min/h) | OR | 95% CI | *P* -value | Cut-off (min/h) | Sensitivity (%) | Specificity (5) | AUC |
|---|---|---|---|---|---|---|---|---|
| All data | | | | | | | | |
| | Rumination | 2.22 | 1.3 - 4.2 | 0.0008 | 19.5 | 51 | 61 | 0.61 |
| | Eating | 1.6 | 1.1 - 2.45 | 0.02 | 11.2 | 53 | 63 | 0.61 |
| Multiparous cows | | | | | | | | |
| | Rumination | 1.8 | 1.1 - 3.45 | 0.03 | 19.3 | 51 | 51 | 0.56 |
| | Eating | 21 | 1.3 - 3.4 | 0.003 | 10.7 | 65 | 59 | 0.65 |
| Primiparous cows | | | | | | | | |
| | Rumination | 3.2 | 1.1 - 11.9 | 0.05 | 18.0 | 68 | 58 | 0.69 |
| | Eating | 1.6 | 0.86 - 3.1 | 0.15 | 11.2 | 46 | 60 | 0.6 |

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# CHAPTER 5 - USE OF ALUMINIZED REFLECTIVE COVERS FOR CALF HUTCHES DURING SUMMER ON CALF HEALTH AND PERFORMANCE ON PREWEANED DAIRY CALVES

## Introduction

Calf rearing is a sensitive constituent of dairy systems, where adequate growth, health, and well-being, are critical components (Windeyer et al., 2014). In extensive areas of the US, calves are challenged by extreme environmental conditions occurring during the hot season, resulting in reduced weight gain and increased morbidity and mortality (Roland et al., 2016). During summer, calves are exposed to thermic stress, defined as a change in the environment causing an alteration in body temperature that is not entirely compensated by thermoregulatory mechanisms (IUPS, 2001).

The adverse effects of extreme heat begin before birth, during the periconceptional period and continue until late gestation, where heat stressed dams produce offspring with lower milk yield during their first lactation, reduced survival, and impaired immunity and metabolism (Carroll et al., 2012; Brown et al., 2016; Guo et al., 2016; Roland et al., 2016). In growing calves, body temperature regulation mechanisms are immature (National Research Council, 2001), resulting in higher susceptibility to changes in ambient conditions (Bateman et al., 2012). Consequently, heat stress may affect behavior, dry matter intake (**DMI)**, average daily gain (**ADG**), rectal temperature, respiratory rate, and disease frequency and survival (Yazdi et al., 2016; Sims et al., 2015; Peña et al., 2016). To address this concern, multiple housing strategies to improve calf cooling by creating more moderate housing microclimates have been evaluated (Spain and Spiers, 1996; Hill et al., 2011; Carter et al., 2014).

Recently, the use of sunlight reflective technology to reduce polyethylene hutch interior temperatures has been proposed (Binion et al., 2014; Carter et al., 2014; Friend et al., 2014). A recent report showed a decrease of about 4ºC inside polyethylene hutches, when aluminized reflective hutch covers (**ARC**) were evaluated in dairies in central Texas (Binion et al., 2014). However, to the authors' knowledge, no evaluations have been performed during the entire pre-weaning period in commercial calf rearing operations, assessing the effectiveness of the expected temperature reduction on improving calf health and performance. Our hypothesis was that the expected change in temperature due to sunlight reflection provided by ARC on polyethylene hutches would indirectly have an effect on the health and performance of pre-weaned dairy calves under hot conditions. Furthermore, resulting lower temperatures would alter calf behavior during the hottest periods of the day. Consequently, our objective was to evaluate the effect of ARC applied on polyethylene hutches on health and performance of pre-weaned dairy calves, during the hot season in Northern Colorado, where summer temperatures can exceed 37ºC (Colorado Climate Center, 2016). Additionally, the effects of ARC on hutch temperature and temperature and humidity index (THI) were evaluated.

## Materials and Methods

### *Study Population and Calf Management*

All the animal related procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee at Colorado State University (Protocol ID: 16-6704AA). This research was conducted from June to October (2016) in a large dairy calf rearing facility, part of a dairy under certified organic management, located in Northern Colorado. Pre-weaned calves were maintained in a total area of approximately 174,000 $m^2$ and housed in

polyethylene hutches (Agri-Plastics, Stoney Creek, ON, Canada) exposed to direct sunlight. Walls built with straw square bales (3 m high) surrounded the area.

Calves were immediately separated from the dam at birth and during the first hour of life had their navel dipped into an iodine 7% solution and were fed 2.8 L of colostrum warmed to 37$^{o}$C. Colostrum feeding was repeated at 3 and 8 hours of life in the maternity facility. The colostrum fed had at least 52 mg/ml of colostrum globulin, as determined by use of colostrometer (Fleenor and Stott, 1980). Within the first day of life, calves were transferred to the calf rearing facilities and housed individually in polyethylene hutches provided with a front yard of 2.25 $m^2$ enclosed by a galvanized welded wire fence and sand bedding. Subsequently, calves received 3.8 L of colostrum fed in a 10-hour interval four times. At 4 d of life, calves had access to small amounts of an organic certified calf starter (16% Organic Calf Starter, Feedex Companies, LLC. South Hutchinsin, KS) that increased according to intake up to 4 - 5 lb/head/day until 56 d of life. A description of this product is presented in Table 15.

Starting at 4 d of life, calves received 2.5 L of pasteurized milk every 12 hours until 14 d of life. From d 15 until d 49 calves received 3 L of milk every 8 hours. At 50 d of life, milk was fed only in the mornings and at 65 d of life, calves were weaned. Water was provided from d 1 in a plastic bucket (8 L) filled twice per day. Dehorning was performed before 30 d of life using electrical cauterization under local anesthesia with veterinary supervision. The vaccination protocol included intranasal Inforce 3 (IBR, PI3, BRSV; Zoetis, Florham Park, NJ ) at 1 d of life, Ultrabac 8 (*Clostridium chauvoei, C. speticum, C. haemoylticum, C. novyi, C. sordelli* and *C. perfringens* type B, C and D; Zoetis, Florham Park, NJ) at 21 d of life, Spirovac L5 (*Leptospira canicola, L. grippotyphosa, L. hardjo, L. icterohaemorrhagiae, and L. pomona* bacterin; Zoetis, Florham Park, NJ) plus a booster of Inforce 3 and Ultrabac 8 at 42 days of life.

**Table 15.** Nutrient composition of the calf starter fed to the study calves from d 3 to d 60 of life (DM basis)

| Nutrient profile[a] | Concentration |
|---|---|
| CP (%) | 16 |
| Fat (%) | 2.4 |
| Fiber (%) | 3.0 |
| ADF (%) | 5.0 |
| Ca (%) | 0.7 - 1.2 |
| P (%) | 0.45 |
| NaCl (%) | 0.2 - 0.7 |
| Mg (%) | 0.2 |
| K (%) | 0.9 |
| Cu (ppm) | 15 |
| Se (ppm) | 0.3 |
| Zn (ppm) | 80 |
| Vitamin A (IU/lb) | 5000 |
| Vitamin D (IU/lb) | 1000 |

[a] Guaranteed analysis provided by the manufacturer (Feedex Companies, South Hutchinson, KS)

In addition to health assessment by the authors, calf health monitoring was performed daily by farm personnel. Calves needing antimicrobial therapy or other drugs not allowed in organic dairy systems were sent to a conventional calf ranch for prompt treatment.

*Experimental Procedures*

Two study periods were included in this research: Study group (**SG**) 1, including 47 calves in each treatment group, monitored from June 30 to September 9, 2016; and SG2 monitored from August 15 to October 14, 2016, with 50 and 51 calves in covered and control hutches, respectively.

Holstein heifers were enrolled at 1 d of life and monitored until 60 d of age. Calves born from dystocia deliveries or with any apparent abnormality were not included. Calves were randomly allocated into two housing treatments using polyethylene hutches that were covered by aluminized reflective covers (covered) or hutches left uncovered (control). Aluminized reflective covers (Cool-Calf Covers, Oceanside, CA, USA) were installed according to manufacturer's directions. Covered hutches had both sidewalls and the roof completely shielded, leaving the back and the front areas of the hutch exposed. Control hutches were not covered at any time. The rear door provided superior airflow for ventilation and its opaque wall reduced the penetration of UV light. Rear ventilations remained open at all times for both treatments throughout the complete study period. Covered and control hutches were located in contiguous blocks of 25 in two parallel lines. Lines were west-east oriented, separated by 5 m, with space of 1.5 between hutches. The hutch front door was south-oriented.

As part of the farm health monitoring program, blood samples were collected from the jugular vein into blood collection tubes without anticoagulant (Becton Dickinson Vacutainer, Franklin Lakes, NJ) between 3 and 7 d of life for determination of serum total protein (**STP**) concentrations to evaluate the status of passive immune transfer. Samples were allowed to clot and

serum was harvested to determine STP concentration using a hand-held refractometer. Concentrations of STP were categorized according to their quartile distribution as low (≤6.5 g/dL); medium (6.6 to 7.4 g/dL); or high (≥7.5 g/dL) to control for initial immune status and dehydration. Calf weight was measured at 1 ± 3 d and at 60 ± 3 d of life using the Weighing Caf-Cart (Raytec, LLC., Ephrata, PA).

To facilitate the analysis, time throughout the day was categorized in 3 hours periods as follows: Day period (DP) 1 = from 00:00 to 02:59 h; DP2 = from 03:00 to 05:59 h; DP3 = from 06:00 to 08:59 h; DP4 = from 09:00 to 11:59 h; DP5 = from 12:00 to 14:59 h; DP6 = from 15:00 to 17:59 h; DP7 = form 18:00 to 20:59 h; and DP8 = from 21:00 to 23:59 h.

*Calf Measurements*

The first outcome measured during farm visits (DP5) performed twice a week was calf behavior (inside or outside of the hutch). Before assessment, it was assured that no other activity was performed within 30 min prior to the evaluation. Next, health status was assessed using the University of Wisconsin calf health-scoring chart (McGuirk, 2008) for screening of fecal, nasal, eye, and ear abnormalities. Scores were categorized as normal (scores1 and 2) and abnormal (scores 3 and 4) as presented by Peña et al. (2016). Additionally, clinical dehydration was evaluated based on the score scale proposed by Walker et al. (1998), defined by eye brightness and location and skin elasticity (normal = bright eyes and skin tent < 2 s; moderate = skin tent > 2 s and eye slightly recess; and severe = skin tent > 10 s and eye are markedly recessed). Finally, the occurrence of spontaneous coughing during the assessment was also recorded.

Rectal temperature and respiratory rate (breaths/min) were measured once per week at the beginning of DP6. Rectal temperature was measured using the GLA M700 rectal thermometer (GLA Agricultural Electronics. San Luis Obispo, CA), whereas respiratory rate was measured by

counting the movements of the abdominal muscles in the flanks during the respiratory cycle before approaching to the hutch.

*Temperature and Humidity Measurements*

Ambient temperature and humidity were measured using two HOBO Pro v2 loggers (Onset Computer Corporation., Bourne MA) set to a sampling rate of 1 reading every 15 min. These loggers were located 3 m high between the hutch lines. In addition, four empty hutches (2 per treatment group) were installed between the lines during both study periods (8 empty hutches in total) to determine inside hutch temperature and THI. The HOBO UX100-011 temp/RH 2.5% loggers (Onset Computer Corporation., Bourne, MA) were installed in the inner surface of the ceiling of the empty hutches, with a sampling rate of 1 reading every 15 min. THI was calculated for ambient and hutch readings using the equation: THI = (1.8 x T + 32) – ((0.55 – 0.0055 x RH) x (1.8 x T – 26)), where T = temperature ($^{o}$C) and RH = relative humidity (Kendall et al., 2008; Vickers et al., 2010). Days were categorized as presenting low or high THI when THI at DP5 < 72 or ≥ 72 units, respectively.

Inner hutch wall and sand bedding temperature were measured consistently at DP5 twice per week in all the hutches housing calves throughout the study, using an IR 1000 infrared thermometer (Klein Tools., Lincolnshire, IL). To measure the temperature of the inner wall, the infrared thermometer was located on the right upper corner of the front door pointing toward the right wall at 30 cm from the floor. To measure sand temperature, the infrared thermometer was pointed toward the center of the bedding inside the hutch. Sand temperatures were measured on shaded dry areas. Gas ammonia concentrations were screened twice per week in all the hutches housing a calf using the Gas-Alert NH3 logger (BW technologies, Schaumburg, IL). Ambient and

hutch temperature and THI were averaged by 3-h period, according to the previously described DP classification.

*Statistical Analysis*

Calf health scores were analyzed using a logistic regression model for repeated measures data (PROC GENMOD, SAS 9.4, SAS institute Inc., Cary, NC), assuming an exchangeable correlation structure. The final model included treatment and rectal temperature. Mortality and culling data were analyzed by use of logistic regression (PROC LOGISTIC), including hutch treatment and study group in the model. Calf rectal temperature and respiratory rate were analyzed using repeated measures (PROC MIXED); the model included treatment, study group, evaluation date and treatment by evaluation date interaction. Time to event analyses (Kaplan-Meier method) were used to evaluate differences in age at the first abnormal health score, at fever presentation, and at clinical dehydration by SG (PROC LIFETEST). The Wilcoxon test was used to determine significant differences between treatment groups and hazard ratios were calculated using Cox proportional hazards models (PROC PHREG). Least square means (LSM) were calculated for ADG and weaning weight using PROC GLM. The final model consisted of treatment and SG and treatment by SG interaction.

The association between calf behavior (inside the hutch at DP5) and treatment was analyzed considering a logistic regression model for repeated measures (PROC GENMOD), assuming an exchangeable correlation structure. The final model included treatment and THI category. ANOVA, (REPEATED statement for PROC GLM) was used to compare empty hutch temperature and THI between housing treatments for both daily average and DP5 – DP6 values. The model included treatment, date, and treatment by date interaction. Temperatures in the inner wall and in the bedding sand were analyzed using repeated measures (PROC GLM). To investigate

the effect of different levels of ambient THI, the model included ambient THI category measured at DP5. The final model included treatment, study group, and THI category, and the treatment by THI category by study group interaction. Statistical significance was defined at $P < 0.05$.

**Results and Discussion**

*Climatic Data*

A summary of ambient THI, temperature, and relative humidity data is presented in Table 16 for both study groups by day period. Overall, temperature, relative humidity, and THI, ranged from -1.3 to 38.4ºC, from 9.8 to 99.7 %, and from 33.6 to 81.1 units, respectively. Day period 5 and DP6 had the highest average THI and temperature, and the lowest relative humidity throughout the study. During SG1, there were 68 days with THI ≥72 units, whereas in SG2 only 33 days had THI ≥72 units.

*Calf Health and Performance*

Calves in both treatments and SGs had similar STP concentrations (assessed between 3 and 7 d of life). Sixteen calves died during the study, but no relationship was found between mortality and treatment ($P = 0.1$). The odds (95% CI) of dying were 8.5 (1.7 – 19.6) times greater for calves in SG1 compared with those in SG2. Sixteen calves that required the use of medical products prohibited in organic certified systems left the farm during the study and no association with the use of ARC ($P = 0.3$) was determined. Reasons for leaving the farm included respiratory disease (72.7%), diarrhea (18.2%) and bloating (9.1%). No differences in the proportions of calves leaving the farm were determined between SG1 and SG2 ($P = 0.09$).

**Table 16.** Summary statistics for ambient THI, temperature, and relative humidity by 3-hour day period in study group 1 and study group 2

| | | Study group 1[a] | | | | Study group 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Day period | Variable | Min | Max | LSM | SE | Min | Max | LSM | SE | *P*-value[b] |
| 1 | THI | 47.3 | 70.2 | 61.3 | 0.2 | 34.2 | 65.7 | 53.3 | 0.2 | <0.0001 |
| | Temperature | 7.7 | 24.0 | 16.7 | 0.2 | 1.0 | 20.8 | 11.5 | 0.2 | <0.0001 |
| | Relative Humidity | 20.7 | 98.4 | 73.5 | 0.6 | 30.4 | 98.1 | 72.5 | 0.6 | 0.9 |
| 2 | THI | 44.3 | 68.8 | 58.3 | 0.2 | 33.3 | 65.1 | 50.8 | 0.2 | <0.0001 |
| | Temperature | 6.6 | 22.6 | 14.7 | 0.2 | -0.5 | 20.1 | 10.0 | 0.2 | <0.0001 |
| | Relative Humidity | 29.7 | 99.1 | 80.7 | 0.6 | 29.7 | 99.1 | 78.6 | 0.6 | 0.4 |
| 3 | THI | 43.2 | 75.2 | 61.4 | 0.2 | 33.3 | 67.7 | 52.1 | 0.2 | <0.0001 |
| | Temperature | 6.0 | 29.2 | 16.8 | 0.2 | -1.3 | 21.9 | 10.8 | 0.2 | <0.0001 |
| | Relative Humidity | 23.9 | 99.6 | 75.6 | 0.6 | 34.0 | 99.7 | 78.1 | 0.6 | 0.2 |
| 4 | THI | 57.4 | 79.6 | 70.4 | 0.2 | 37.2 | 76.6 | 62.6 | 0.2 | <0.0001 |
| | Temperature | 14.0 | 34.9 | 24.2 | 0.2 | 2.0 | 32.9 | 18.3 | 0.2 | <0.0001 |
| | Relative Humidity | 12.4 | 93.7 | 51.3 | 0.6 | 12.4 | 99.7 | 55.8 | 0.6 | <0.0001 |
| 5 | THI | 62.7 | 80.5 | 74.3 | 0.2 | 40.5 | 77.7 | 68.5 | 0.2 | <0.0001 |
| | Temperature | 17.5 | 37.3 | 28.9 | 0.2 | 3.9 | 34.3 | 24.1 | 0.2 | <0.0001 |
| | Relative Humidity | 11.3 | 76.8 | 34.7 | 0.6 | 10.7 | 87.8 | 35.3 | 0.6 | 1.00 |
| 6 | THI | 53.8 | 81.1 | 74.0 | 0.2 | 43.1 | 78.3 | 68.7 | 0.2 | <0.0001 |
| | Temperature | 12.0 | 38.4 | 29.1 | 0.2 | 5.5 | 34.1 | 24.5 | 0.2 | <0.0001 |
| | Relative Humidity | 9.3 | 88.9 | 32.9 | 0.6 | 9.9 | 87.2 | 32.6 | 0.6 | 1.00 |
| 7 | THI | 53.3 | 80.0 | 69.9 | 0.2 | 38.8 | 75.8 | 63.8 | 0.2 | <0.0001 |
| | Temperature | 11.5 | 37.5 | 24.4 | 0.2 | 3.3 | 31.4 | 19.5 | 0.2 | <0.0001 |
| | Relative Humidity | 9.3 | 88.6 | 45.8 | 0.6 | 17.0 | 91.7 | 45.8 | 0.6 | 1.00 |
| 8 | THI | 51.8 | 76.1 | 65.0 | 0.2 | 36.2 | 70.0 | 57.6 | 0.2 | <0.0001 |
| | Temperature | 10.7 | 32.1 | 19.7 | 0.2 | 2.1 | 25.7 | 14.4 | 0.2 | <0.0001 |
| | Relative Humidity | 14.3 | 95.2 | 62.1 | 0.6 | 21.4 | 96.3 | 62.1 | 0.6 | 1.00 |

[a]Study group 1 was monitored from June 30 to September 9, 2016; study group 2 from August 15 to October 14, 2016.

[b]Statistical significance for the comparison of THI, temperature, and relative humidity least squares means between the study groups 1 and 2 within day period.

Results from the logistic regression analyses for health scores are presented in Table 17. Occurrence of diarrhea and housing treatment were associated; the odds (95% CI) of presenting diarrhea were 1.30 (1.01-1.60) times greater for calves housed in covered hutches than for those in the control group. Similarly, the odds of abnormal ear scores were 1.40 (1.03-2.00) times greater in calves in covered hutches compared to calves in control hutches. No significant associations were found between housing treatment and the occurrence of clinical dehydration, and nasal and eye discharge. As expected, the occurrence of abnormal health scores was associated with of high rectal temperature and spontaneous coughing. A one-unit increase in rectal temperature increased the odds (95% CI) of diarrhea, clinical dehydration, nasal discharge, eye discharge, and dropped ears by 1.4 (1.3 – 1.6), 1.7 (1.2 – 2.2), 1.3 (1.1 – 1.5), 1.2 (1.0 – 1.4) and 1.5 (1.3 – 1.7), respectively. Spontaneous coughing was associated with nasal discharge increasing the odds of an abnormal nasal score by 1.9 (1.3 – 2.7) on calves coughing.

Rectal temperatures were similar for calves housed in covered and in control hutches (Figure 40) and the interaction between treatment and evaluation date was not significant. Overall, calves in SG2 had lower average rectal temperature than those in SG1 (39.2 ± 0.02 and 39.1 ± 0.02ºC, $P = 0.01$). Similarly, respiratory rates were similar for calves housed in covered and control hutches (61 ± 1 and 60 ± 1 breaths/min) and between study groups. However, the repeated measures analyses indicated a significant interaction effect between treatment and date of evaluation ($P = 0.04$ Figure 41). In order to perform time-to-event analyses for the first case of an abnormal health score the data set was divided by study group, as both groups were not contemporaneous in the assessment dates. No differences in the survival functions were determined for diarrhea between treatments in both study groups (SG1 $P = 0.6$; SG2 $P = 0.2$).

**Table 17.** Odds ratios for health score assessment indicative of health problems for calves housed in covered compared to calves housed in control hutches

| Health variable | Odds Ratio | 95% CI[a] | *P*-value |
|---|---|---|---|
| Diarrhea[b] | 1.30 | 1.01-1.60 | <0.05 |
| Clinical- Dehydration[c] | 0.70 | 0.30-1.30 | 0.2 |
| Nasal[d] | 1.30 | 1.00-1.80 | 0.10 |
| Eyes[e] | 1.46 | 0.90-2.20 | 0.06 |
| Ears[f] | 1.40 | 1.03-2.00 | <0.05 |

[a]95% confidence interval.
[b]Refers to calves with pasty, loose and watery feces.
[c]Indicates calves with delayed skin elasticity and dry and recessed eyes.
[d]Comprises calves showing unilateral or bilateral mucus or mucopurulent nasal discharge.
[e] Includes calves with unilateral or bilateral eye discharge or crusty eyes.
[f]Calves presenting unilateral or bilateral droopy ears and/or head tilt.

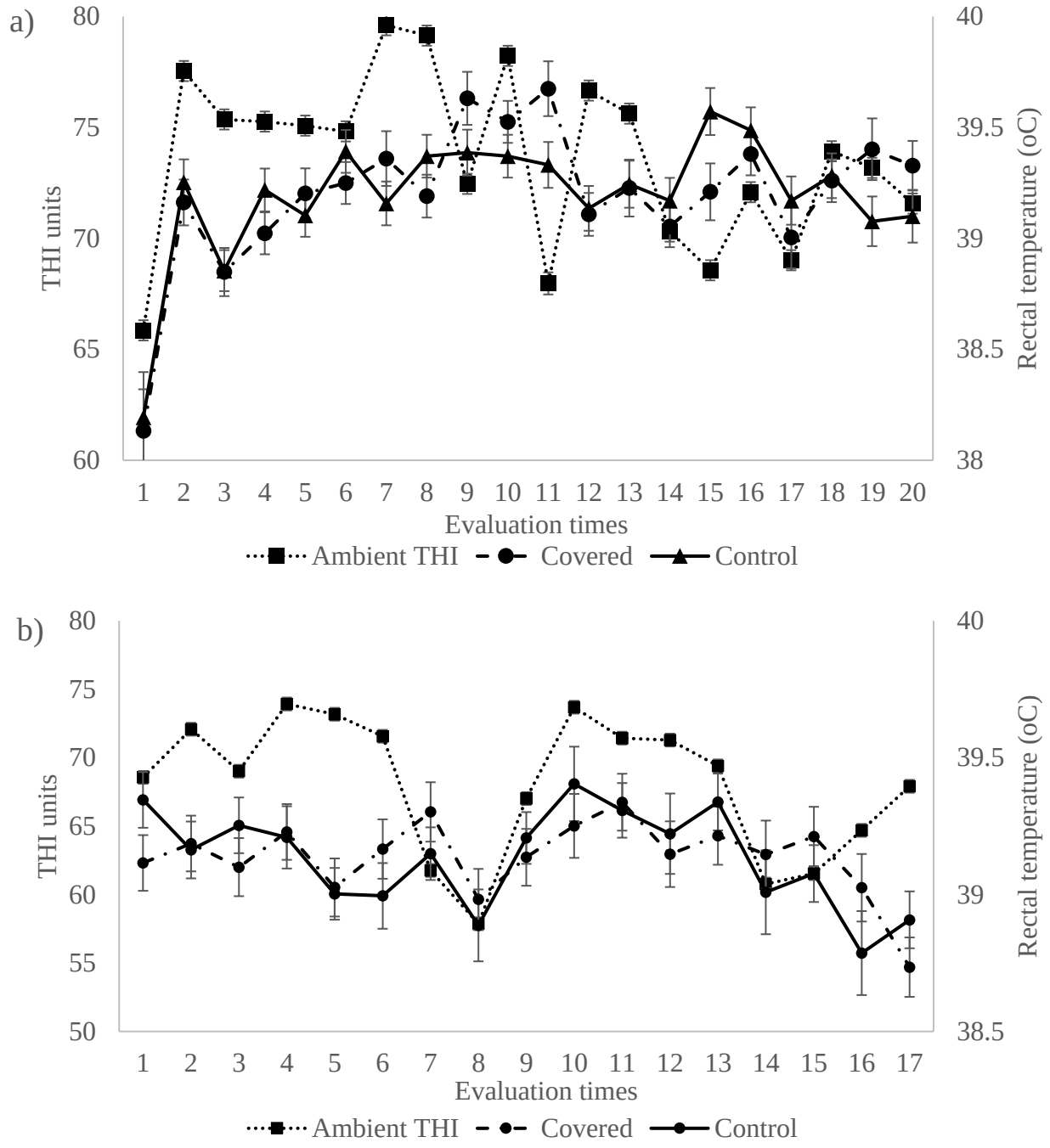



**Figure 40.** Ambient THI at DP6 (dotted line) and rectal temperature in calves housed in covered (dashed line) or control hutches (solid line) in study groups 1 (a) and 2 (b), during weekly evaluations. Study group 1 was monitored from June 30 to September 9, 2016; study group 2 from August 15 to October 14, 2016

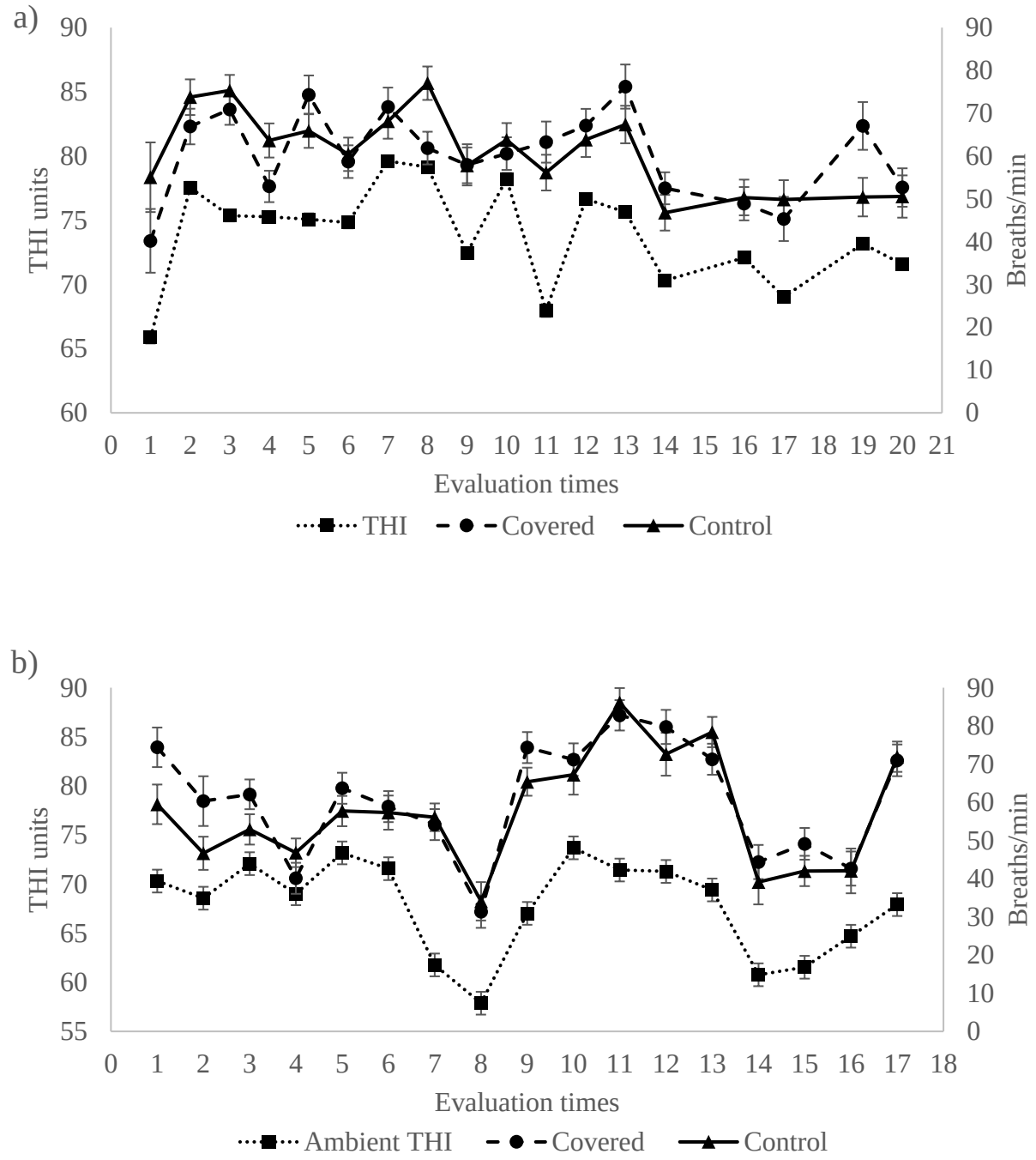



**Figure 41.** Variation in ambient THI at DP6 (dotted line) and respiratory rate in calves housed in covered (dashed line) or control hutches (solid line) in study groups 1 (a) and 2 (b), during weekly evaluations. Study group 1 was monitored from June 30 to September 9, 2016; study group 2 from August 15 to October 14, 2016

Regarding time to first diagnosis of nasal abnormalities, only calves in SG2 evidenced significant differences between hutch treatments ($P$ <0.001); the Kaplan-Meier median time was 8 d vs. 22 d in covered vs. control hutches. In agreement, in SG2 the hazard of nasal discharge was 2.1 times greater on calves in covered hutches compared to those in control hutches. No differences between hutch treatments were determined for the time to diagnosis of ear abnormalities, occurrence of fever, or clinical dehydration.

Calf ADG was similar for both hutch treatments within study groups (Table 18). Nonetheless, calves in SG2 had significantly greater ADG and weaning weight than calves in SG1 (1.4 ± 0.02 vs. 1.25 ± 0.01 kg, $P$ <0.0001; and 80.9 ± 0.9 vs. 75.2 ± 1.03 Kg, $P$ <0.0001, respectively). There were no differences in ADG and weaning weight by STP levels.

Overall, the proportions of calves found inside the hutches at DP5 were 64% and 52% when THI ≥72 and THI < 72 units, respectively. The logistic regression analysis indicated that treatment and THI category were significantly associated with this behavior ($P$ = 0.03 and $P$ < 0.0001, respectively). The odds of remaining inside the hutch were 1.33 (1.03 - 1.70) times greater for calves housed in control hutches than for those in the covered hutches. Calves evaluated during high THI category had 2.0 (1.67 - 2.3) times greater odds of remaining inside the hutch than calves exposed to low THI category.

*Hutch Temperature and THI*

Average temperature in empty hutches during SG1 was higher in covered compared to control hutches (23.2 ± 0.06 vs. 22.8 ± 0.06 $^{o}$C, P<0.0001). In agreement, THI was also higher in covered hutches (68.6 ± 0.06 vs. 67.6 ± 0.06 units, $P$ < 0.001). Similarly, during SG2, average temperature and THI were higher in covered compared to controls hutches: 17.1 ± 0.07 vs. 16.9 ± 0.07$^{o}$C ($P$ = 0.01) and 60.2 ± 0.08 vs. 59.6 ± 0.08 units ($P$ <0.001), respectively.

**Table 18.** Least square means ± SE for birth weight, weaning weight, ADG and serum total protein within the study groups according to treatment (covered vs. control hutches)

| | Variable | Covered | Control | *P*-value |
|---|---|---|---|---|
| Study group 1[a] | | | | |
| | Birth weight (Kg) | 41.5 ± 0.7 | 41.5 ± 0.7 | 0.9 |
| | Weaning weight (Kg) | 77.0 ± 1.6 | 73.4 ± 1.4 | 0.3 |
| | ADG (Kg) | 1.3 ± 0.03 | 1.2 ± 0.02 | 0.4 |
| | Serum total protein (g/dL) | 7.3 ± 0.1 | 7.3 ± 0.1 | 0.9 |
| Study group 2 | | | | |
| | Birth weight (Kg) | 38.8 ± 0.7 | 40.2 ± 0.6 | 0.2 |
| | Weaning weight (Kg) | 80.5 ± 1.4 | 81.2 ± 1.3 | 0.9 |
| | ADG (Kg) | 1.4 ± 0.02 | 1.4 ± 0.02 | 0.9 |
| | Serum total protein (g/dL) | 6.9 ± 0.1 | 6.9 ± 0.1 | 0.9 |

[a]Study group 1 was monitored from June 30 to September 9, 2016; Study group 2 from August 15 to October 14, 2016

These differences in temperature and THI were sustained throughout the study period (Figure 42). A summary of hutch temperature, THI, and relative humidity in covered and control empty hutches by DP and study group is presented in Table 19. Interestingly, when the analyses were restricted to values recorded at DP5 and DP6, the hottest DPs during the monitoring period, hutch temperature and THI did not differ between hutch treatments at SG1 (Temperature: 32.0 ± 0.05 vs. 32.0 ± 0.05 $^{o}$C, $P$ = 0.7; THI: 76.8 ± 0.04 vs. 76.7 ± 0.05 units, $P$ = 0.6). Contrasting, at SG2 hutch temperature and THI were lower in covered compared to control hutches (Temperature: 25.6 ± 0.05 vs. 26.1 ± 0.05$^{o}$C, $P$ <0.001; THI: 69.8 ± 0.04 vs. 70.3 ± 0.04 units, $P$ <0.001). Covered hutches had significantly higher temperature and THI during DP 1, 2, 3, 7 and 8 in both study groups.

For hutches housing calves, the average inner wall temperature was lower in covered compared to control hutches in both study groups (Figure 43a). Overall, covered hutches had a temperature of 24.4 ± 0.13$^{o}$C vs. 25.4 ± 0.13$^{o}$C in control hutches ($P$ < 0.0001). As expected, in days with ambient THI ≥72 units, the inner wall temperature was significantly higher compared to days with low THI (28.7 ± 0.11 vs. 21.1 ± 0.14$^{o}$C, $P$ <0.0001). In agreement, inner wall temperature was lower in SG2 compared to SG1 (23.9 ± 0.11 vs. 25.8 ± 0.14$^{o}$C, $P$ <0.0001).

The triple interaction term between treatment, study group, and THI level was significant ($P$ <0.0001; Table 20). Inner wall temperature was lower in covered compared to control hutches when the THI exceeded 72. However, there were no differences between treatments, and between study groups when the ambient THI was lower than 72 (Table 20). Sand bedding temperature did not differ between covered and control hutches (21.1 ± 0.12 vs. 21.4 ± 0.12$^{o}$C, $P$ = 0.07; Figure 43b).

**Table 19.** Summary of average THI, temperature, and relative humidity measured in covered and control empty hutches allocated within the experimental hutch lines during the study.

| | | Study group 1[a] | | | | | Study group 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Covered (n=4) | | Control (n=4) | | | Covered (n=4) | | Control (n=4) | | |
| Day period | Variable | LSM | SE | LSM | SE | *P*-value[b] | LSM | SE | LSM | SE | *P*-value |
| 1 | THI | 61.9 | 0.1 | 60.2 | 0.1 | <0.0001 | 53.2 | 0.2 | 51.4 | 0.2 | <0.0001 |
| | Temperature | 17.1 | 0.1 | 15.8 | 0.1 | <0.0001 | 11.4 | 0.1 | 10.4 | 0.1 | <0.0001 |
| | Relative Humidity | 74.3 | 0.4 | 78.7 | 0.4 | <0.0001 | 73.1 | 0.4 | 77.5 | 0.4 | <0.0001 |
| 2 | THI | 59.2 | 0.1 | 57.2 | 0.1 | <0.0001 | 50.8 | 0.2 | 49.1 | 0.2 | <0.0001 |
| | Temperature | 15.2 | 0.1 | 14 | 0.1 | <0.0001 | 10.0 | 0.1 | 9.1 | 0.1 | <0.001 |
| | Relative Humidity | 80.6 | 0.4 | 85.5 | 0.4 | <0.0001 | 78.5 | 0.4 | 82.9 | 0.4 | <0.001 |
| 3 | THI | 62.9 | 0.1 | 62.5 | 0.1 | 0.9 | 52.8 | 0.2 | 52.3 | 0.2 | 0.9 |
| | Temperature | 17.9 | 0.1 | 17.7 | 0.1 | 0.9 | 11.3 | 0.1 | 11.1 | 0.1 | 0.9 |
| | Relative Humidity | 72.1 | 0.4 | 73.7 | 0.4 | 0.5 | 75.7 | 0.4 | 77.9 | 0.4 | 0.1 |
| 4 | THI | 73.7 | 0.1 | 74.3 | 0.1 | 0.3 | 65.2 | 0.2 | 66.5 | 0.2 | <0.0001 |
| | Temperature | 27.6 | 0.1 | 28.3 | 0.1 | <0.01 | 20.7 | 0.1 | 21.9 | 0.1 | <0.0001 |
| | Relative Humidity | 42.6 | 0.4 | 40.9 | 0.4 | 0.4 | 47.9 | 0.4 | 45.66 | 0.4 | 0.1 |
| 5 | THI | 77.6 | 0.1 | 77.6 | 0.1 | 0.9 | 70.6 | 0.2 | 71.2 | 0.2 | 0.5 |
| | Temperature | 32.7 | 0.1 | 32.7 | 0.1 | 0.9 | 26.3 | 0.1 | 27 | 0.1 | 0.04 |
| | Relative Humidity | 29.3 | 0.4 | 28.9 | 0.4 | 0.9 | 31.1 | 0.4 | 30.1 | 0.4 | 0.9 |
| 6 | THI | 75.9 | 0.1 | 75.8 | 0.1 | 0.9 | 69.2 | 0.2 | 69.5 | 0.2 | 0.9 |
| | Temperature | 31.2 | 0.1 | 31.2 | 0.1 | 0.9 | 25.0 | 0.1 | 25.4 | 0.1 | 0.9 |
| | Relative Humidity | 30.8 | 0.4 | 30.7 | 0.4 | 0.9 | 32.3 | 0.4 | 32.1 | 0.4 | 0.9 |
| 7 | THI | 70.4 | 0.1 | 69.7 | 0.1 | 0.04 | 62.8 | 0.2 | 61.6 | 0.2 | <0.0001 |
| | Temperature | 24.8 | 0.1 | 24.1 | 0.1 | 0.005 | 18.6 | 0.1 | 17.6 | 0.1 | <0.0001 |
| | Relative Humidity | 46.5 | 0.4 | 48.0 | 0.4 | 0.7 | 48.9 | 0.4 | 51.5 | 0.4 | 0.01 |
| 8 | THI | 65.4 | 0.1 | 64.1 | 0.1 | <0.0001 | 57.1 | 0.2 | 55.5 | 0.2 | <0.0001 |
| | Temperature | 19.8 | 0.1 | 18.9 | 0.1 | <0.0001 | 14.0 | 0.1 | 12.9 | 0.1 | <0.0001 |
| | Relative Humidity | 63.6 | 0.4 | 66.7 | 0.4 | <0.0001 | 63.9 | 0.4 | 67.8 | 0.4 | <0.0001 |
| Average | THI | 68.6 | 0.06 | 67.6 | 0.06 | <0.0001 | 60.2 | 0.08 | 59.6 | 0.08 | <0.0001 |
| | Temperature | 23.2 | 0.06 | 22.8 | 0.06 | <0.0001 | 17.1 | 0.07 | 16.9 | 0.07 | <0.0001 |
| | Relative Humidity | 54.9 | 0.20 | 56.5 | 0.20 | <0.0001 | 56.7 | 0.20 | 58.4 | 0.20 | <0.0001 |

[a]Study group 1 was monitored from June 30 to September 9, 2016; study group 2 from August 15 to October 14, 2016.

[b]Statistical significance for the comparison of THI, temperature, and relative humidity least squares means between covered and control hutches within day period and study group

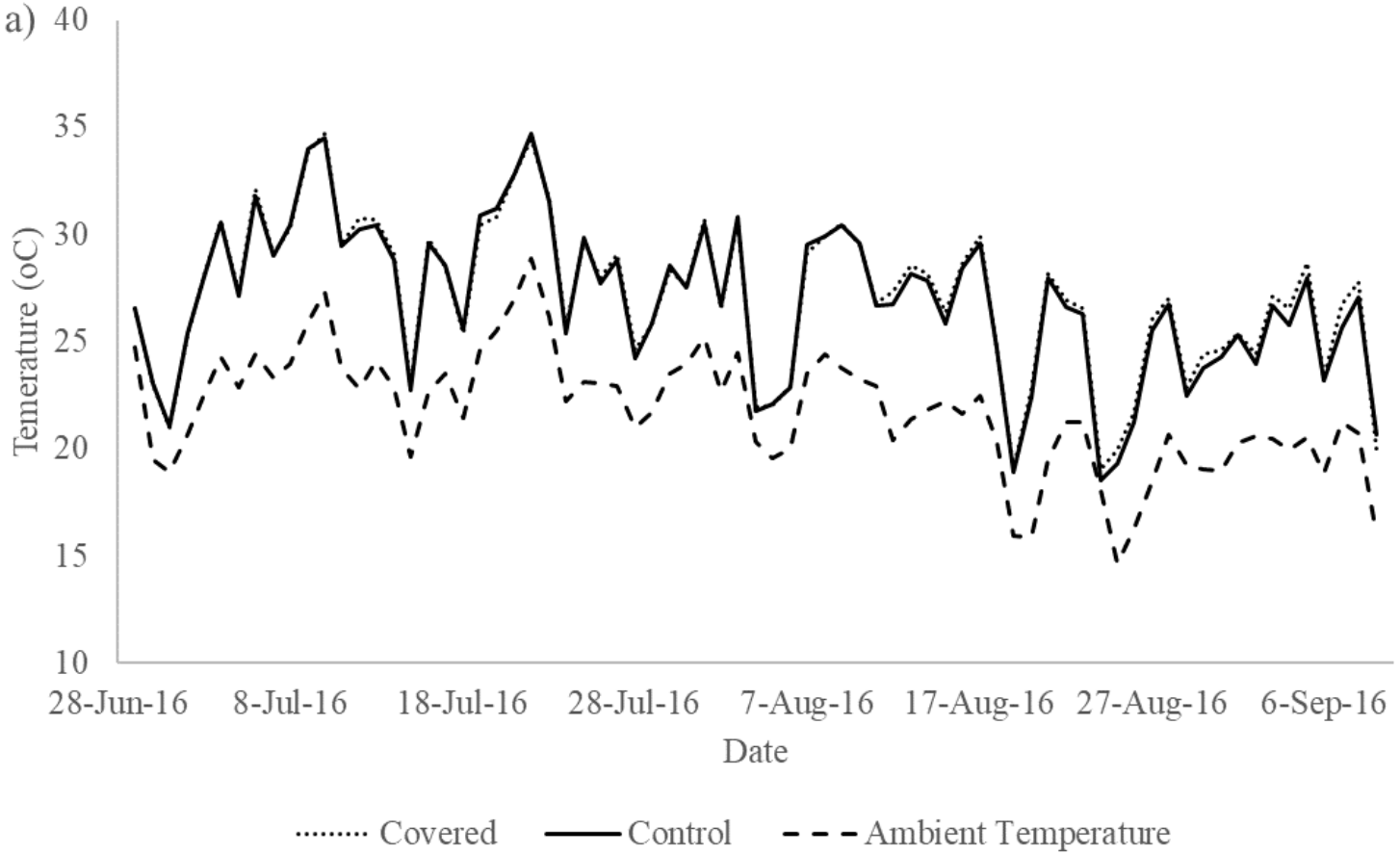
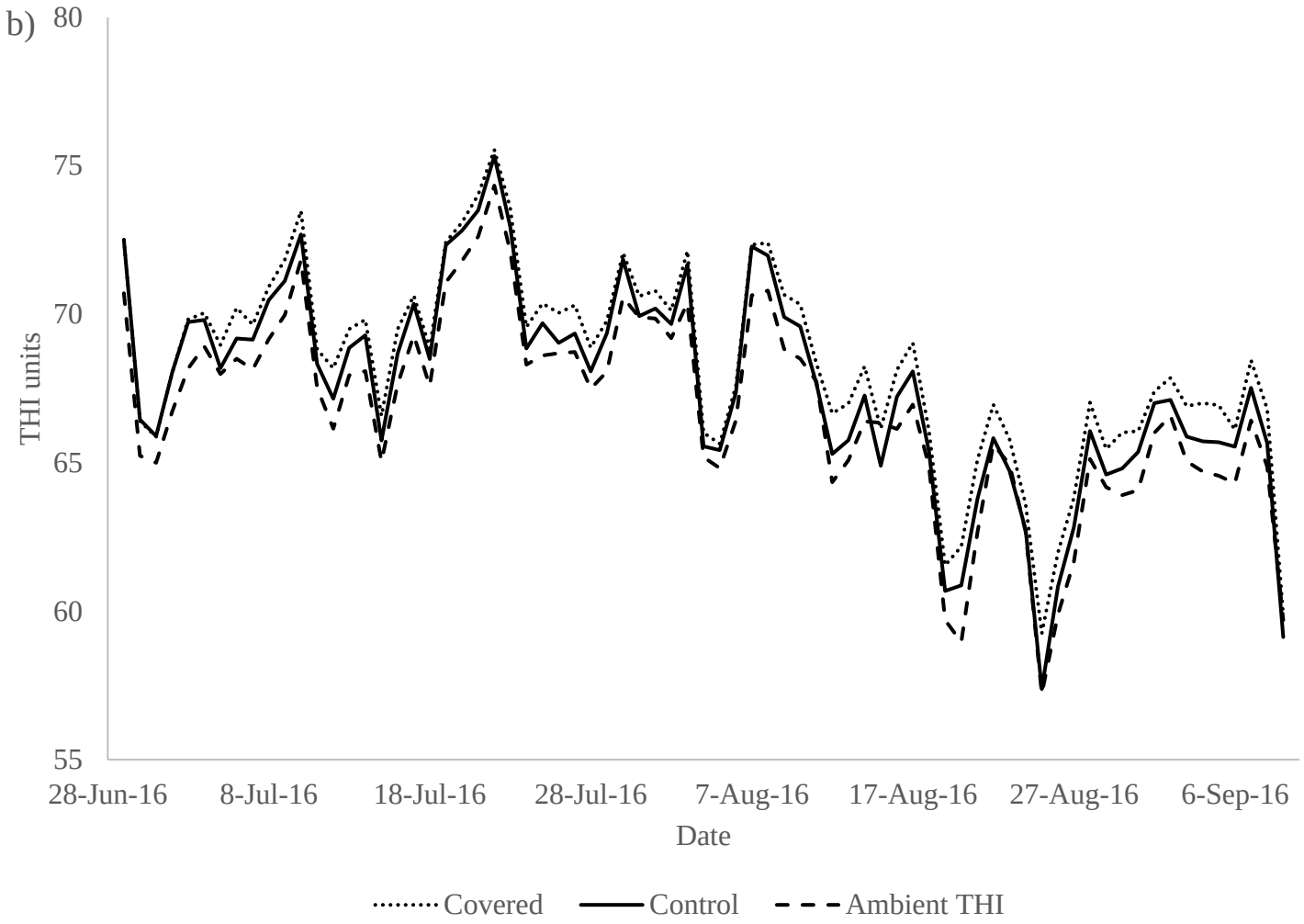
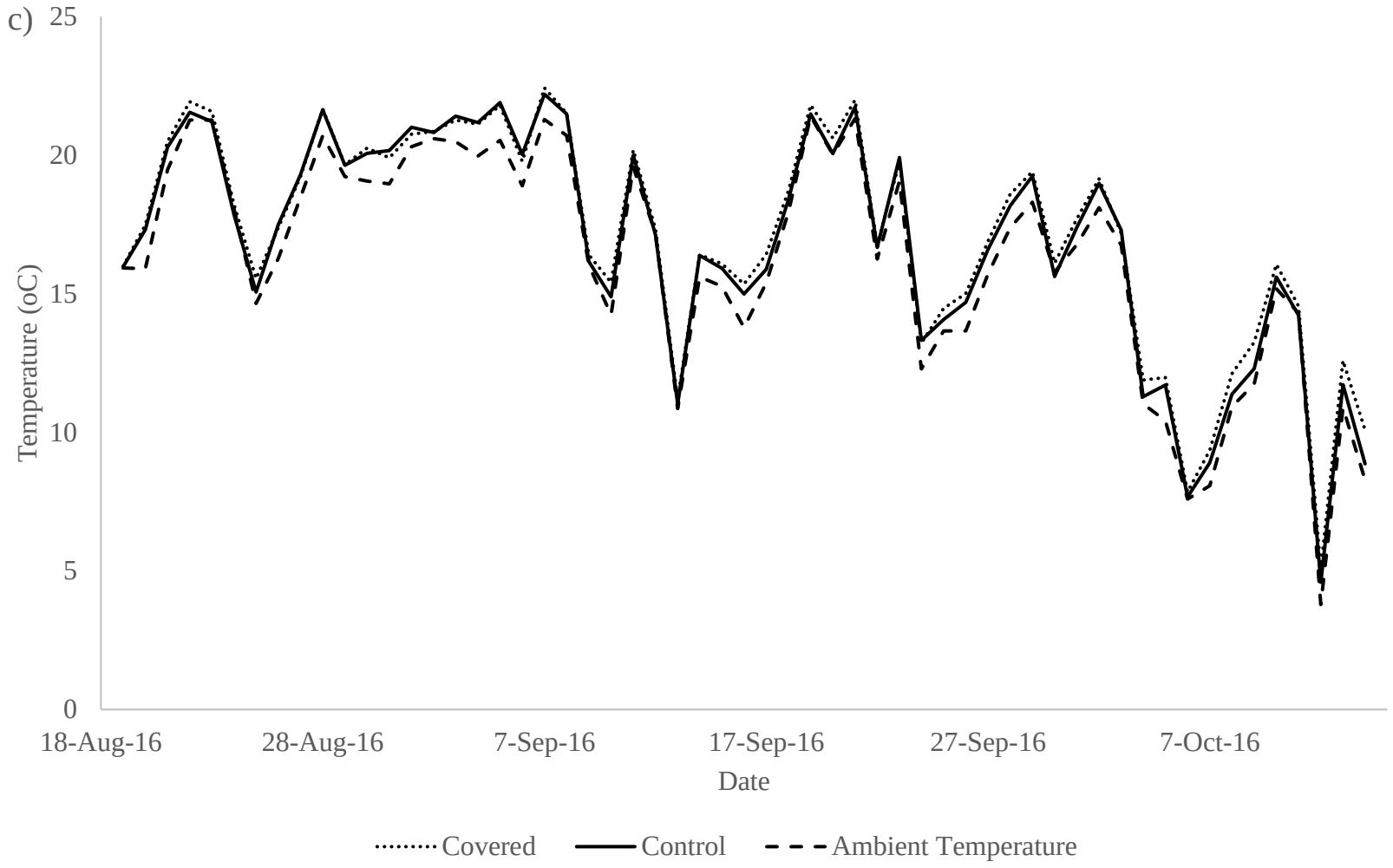
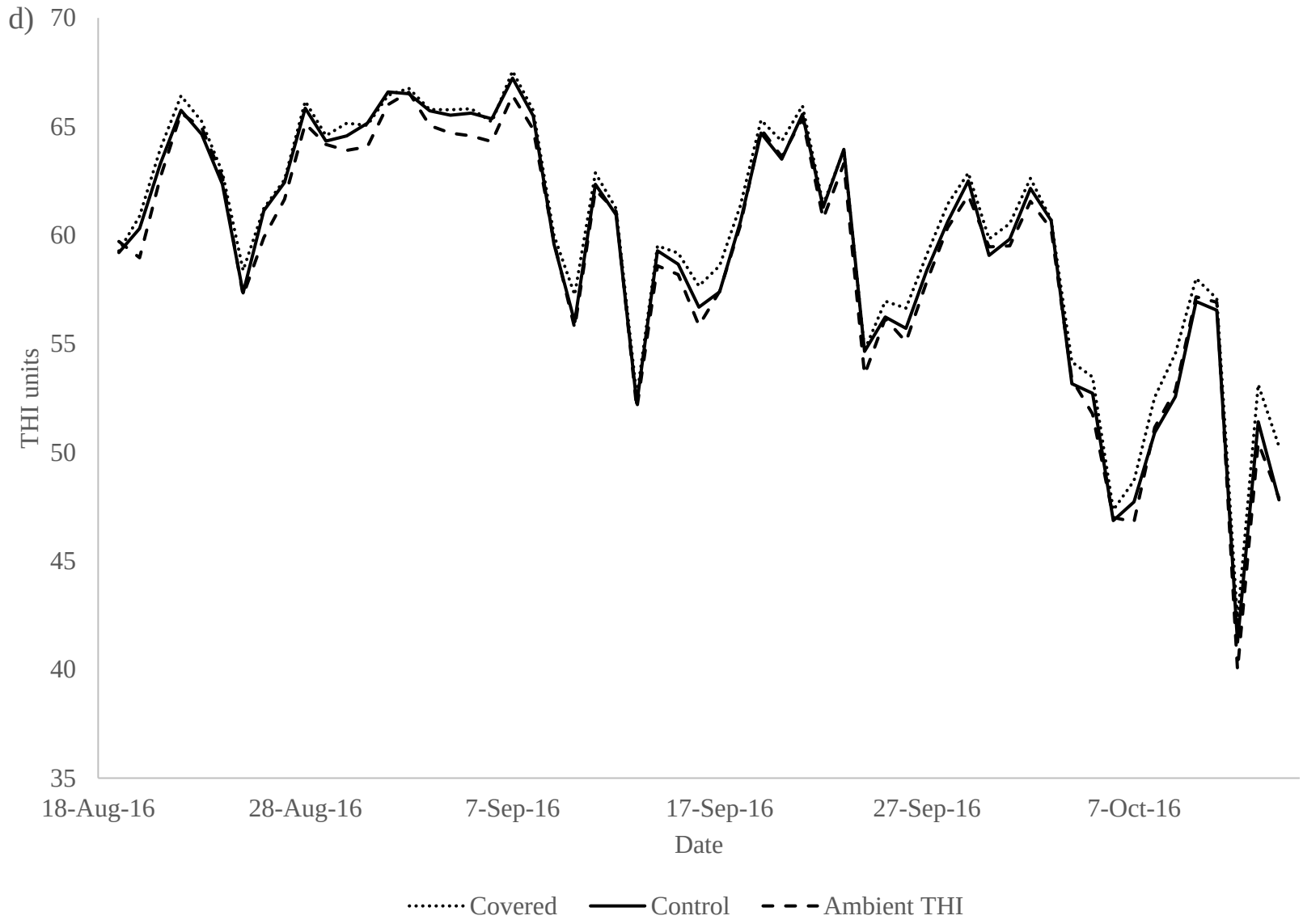



**Figure 42.** Average daily hutch temperature and THI in empty hutches. Study group 1: a) temperature in covered hutches (n = 2, dotted line), control hutches (n = 2, solid line), and ambient temperature (dashed line). b) THI in covered hutches (n = 2, dotted line), control hutches (n = 2, solid line), and ambient temperature (dashed line). Study group 2: c) temperature in covered hutches (n = 2, dotted line), control hutches (n = 2, solid line), and ambient temperature (dashed line). d) THI in covered hutches (n = 2, dotted line), control hutches (n = 2, solid line), and ambient temperature (dashed line). Study group 1 was monitored from June 30 to September 9, 2016; study group 2 from August 15 to October 14, 2016


b)
80
75
70
65
60
55
THI units
28-Jun-16
8-Jul-16
18-Jul-16
28-Jul-16
7-Aug-16
17-Aug-16
27-Aug-16
6-Sep-16
Date
Covered
Control
Ambient THI


c)
25
20
15
10
5
0
Temperature (oC)
18-Aug-16
28-Aug-16
7-Sep-16
17-Sep-16
27-Sep-16
7-Oct-16
Date
Covered
Control
Ambient Temperature


d)
70
65
60
55
50
45
40
35
THI units
18-Aug-16
28-Aug-16
7-Sep-16
17-Sep-16
27-Sep-16
7-Oct-16
Date
Covered
Control
Ambient THI

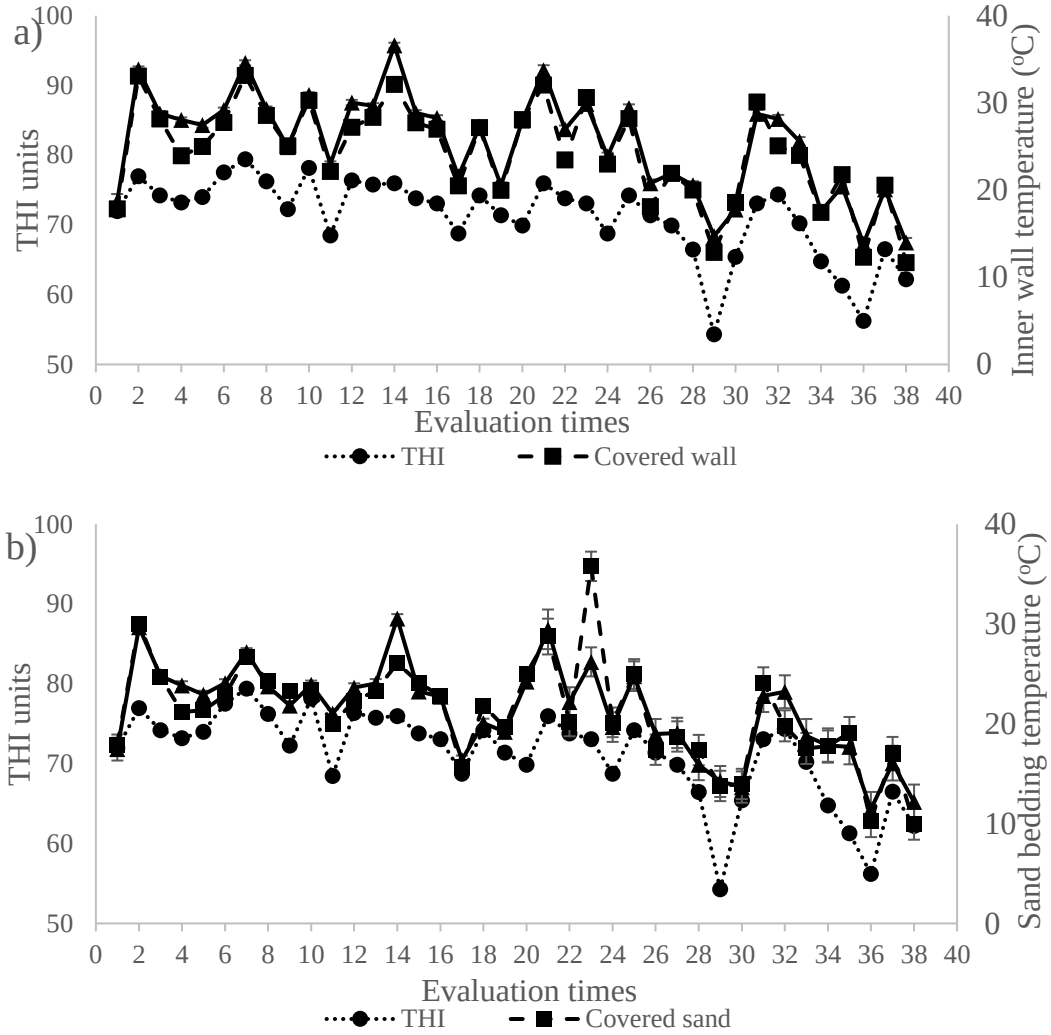



**Figure 43.** a) Inner wall temperature in covered (dashed line) or control hutches (solid line) housing calves. b) Fluctuation of the sand bedding temperature in covered (dashed line) and control hutches (solid line). Ambient THI at DP 5 is presented with a dotted line. Evaluation times from 1 to 20 and from 21 to 38 consider observations from study group 1 and t 2 respectively. Study group 1 was monitored from June 30 to September 9, 2016; study group 2 from August 15 to October 14, 2016

**Table 20.** Temperatures of the inner wall and sand bedding at different THI categories (High: THI ≥ 72 units, low: THI < 72 units) measured at day period 5 (12:00 h – 2:59 h) on covered and control hutches housing calves. Results are presented by study group.

| | Study group 1[a] | | | | | Study group 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Wall temperature (°C) | | | | | Wall Temperature (°C) | | | | |
| THI level[b] | Covered | SE | Control | SE | *P*-value | Covered | SE | Control | SE | *P*-value |
| ≥72 | 28.1 | 0.17 | 30.0 | 0.17 | <0.001 | 28.0 | 0.27 | 29.2 | 0.21 | <0.05 |
| <72 | 22.4 | 0.37 | 23.1 | 0.36 | 0.8 | 19.1 | 0.13 | 19.6 | 0.19 | 0.6 |
| | Sand Temperature (°C) | | | | | Sand Temperature (°C) | | | | |
| THI level | Covered | SE | Control | SE | *P*-value | Covered | SE | Control | SE | *P*-value |
| ≥72 | 23.8 | 0.17 | 24.3 | 0.16 | 0.9 | 24.1 | 0.25 | 24.5 | 0.26 | 0.9 |
| <72 | 20.0 | 0.35 | 20.0 | 0.99 | 0.9 | 16.6 | 0.17 | 16.6 | 0.18 | 0.9 |

[a]Study group 1 was monitored from June 30 to September 9, 2016; Study group 2 from August 15 to October 14, 2016

[b]THI determined considering ambient temperature and relative humidity provided by loggers located between the hutch lines

As expected, when ambient daily THI ≥72 units, sand temperature was higher in covered hutches (24.17 ± 0.12 vs. 18.25 ± 0.14$^{o}$C, $P$ <0.0001). In agreement, sand temperatures were higher in SG1 compared to SG2 (21.98 ± 0.13 and 20.44 ± 0.11$^{o}$C, $P$ <0.0001). Similar to the inner wall temperature model, the triple interaction term was significant ($P$ <0.0001). However, there were no differences between covered and control hutches within THI category.

Most of the assessments for gas ammonia indicated concentrations below the detection levels and consequently no statistical testing was performed.

Extreme heat is recognized as an important stressor affecting the welfare and health of dairy cattle. Several strategies regarding housing and cooling have been evaluated (Hill et al., 2011; Binion et al., 2014; Peña et al., 2016), considering disease presentation, performance, rectal temperature, and respiratory rate as indicative parameters of heat stress (Perano et al., 2015; Nabenishi and Yamazaki, 2016; Peña et al., 2016).

The use of polyethylene hutches in calf rearing is widespread in the US, with approximately 900.000 units in place (Binion et al., 2014). However, high temperature and relative humidity in this type of hutches during summer months have been documented as a potential concern (Hill et al., 2011; Peña et al., 2016).

The use of sunlight reflection technology has recently been proposed to mitigate heat conditions inside polyethylene hutches and the effect of ARC on interior temperature (Binion et al., 2014; Binion and Friend, 2015), ADG, and calf behavior (Carter et al., 2014) has been evaluated. However, to our knowledge, the effect of ARC use on calf health through the complete pre-weaning period has not been reported. Our study centered on the occurrence of clinical symptoms present in the main diseases during this phase of calf rearing. Our results indicated associations between housing treatment and some of the health scores evaluated. Contrary to what

may be anticipated, the probabilities of abnormal fecal and ear scores were greater in the covered group. Although the magnitude of the odds ratios (Table 17) for the occurrence of abnormal fecal and ear scores does not indicate a strong association between hutch treatment and disease occurrence, these results are in line with greater average temperature and THI found in this group of hutches. The resulting coverage of the openings designed for ventilation on the apex of the roof by the ARC and warmer inside temperatures during some periods of the day (Table 16) could be a potential explanation for this finding.

Calves in both housing treatments were exposed to high air temperatures, which reduces the efficiency of sweating and panting for evaporative cooling (West, 2003). Calves in SG1 had greater exposure to higher temperatures and THI during longer periods compared to SG2, which could explain their lower health and growth performance. During the monitoring period, about 70% of the calves presented at least one abnormal health score, reflecting the exposure to challenging environmental conditions in both treatment groups. These values are in the upper range of disease incidence reported in previous studies (Windeyer et al., 2012; Sims et al., 2015; Peña et al., 2016). However, in the current study, calves were systematically assessed for multiple health scores, which may not be comparable to previous reports based on the number of calves that required medical treatment. In addition, due to treatment restrictions associated with organic certification, sick calves showing no signs of significant recovery were submitted to an external calf ranch for conventional treatment.

Hot weather conditions are associated with compromised calf ability to absorb immunoglobulins (West, 2003) and with an affected composition and content of IgG in the colostrum produced by the dams (Quigley and Drewry, 1998; West, 2003). Calves in this study evidenced adequate levels of STP. However, as hot conditions increase calf dehydration altering

STP concentration, the ability to indirectly predict the success of the passive immunity transfer using this parameter may be reduced. Although it is accepted that STP concentrations > 5.2 g/dL indicate adequate IgG concentrations on calves (Windeyer et al., 2014), STP concentrations are not always associated with IgG concentration > 1000 mg/dL, which is generally considered an adequate concentration (McGuirk et al., 2004; Foster et al., 2006). Peña et al. (2016) reported average STP concentrations of 6.05 ± 0.14 g/dL on calves housed in polyethylene hutches; our average STP was 7.1 ± 0.07 g/dL. Apparently, both results represent a satisfactory passive immunity transfer. However, in our study, STP levels were not associated with the likelihood of mortality and culling before 60 d of life, suggesting that baseline values for STP as a measure of immunocompetence may need adjustments that consider environmental conditions that favor the occurrence of dehydration.

Average daily weight gain ranged from 1.2 to 1.4 kg. Calves included in SG2 had significantly greater ADG and weaning weight, which may be in part explained by exposure to milder temperature and THI levels (Tables 2 and 6). However, when the effect of ARC was investigated no differences were found in both study groups, which is in agreement with the results from Carter et al. (2014), where ADG assessed in 38 calves housed in reflective insulated hutches did not differ with controls at 32 and 52 d of life in the Panhandle region of Texas.

Rectal temperatures found in our study were comparable to those reported by Lammers et al. (1996) for calves of similar age (39.6 ± 0.05$^{o}$C) during summer in Northern US climate. Throughout the monitoring period, ARC did not affect rectal temperature at both low (<72) or high (≥72) ambient THI. Conversely, other housing treatments have shown significant improvements on rectal temperature. For example, Peña et al. (2016) found that rectal temperatures significantly decreased when calves were placed in wired fence hutches under shade compared to those in

polyethylene hutches, when rectal temperature was measured at 15:00 h. However, in that study no differences were found when temperature was measured at 09:00 h, suggesting that the hottest periods of the day should be considered for the assessment of rectal temperature during summer, as they provide higher exposure to severe ambient conditions. Rectal temperature was associated with the occurrence of diarrhea, clinical dehydration, nasal discharge, eye discharge, and dropped ears, indicating a reliable evaluation of health scores. Similarly, spontaneous coughing was associated with nasal discharge increasing the odds of an abnormal nasal score by 1.9 (1.3 – 2.7) on calves coughing. Consequently, their assessment could be considered when the effect of housing strategies on calf health is evaluated

Respiratory rates are indicated to be more sensitive to ambient THI than body temperature (Berman, 2005). Contrary to other cooling strategies, such as the use of fans, shade and passive ventilation (West, 2003; Hill et al., 2011; Peña et al., 2016), the use of ARC had no effect reducing this parameter when compared to control hutches, likely due to the inability of the ARC to create a microclimate with significantly lower temperature at time of our evaluation (DP5).

Health, performance, behavior, body temperature and respiratory rate are influenced by ambient conditions (West, 2003; Roland et al., 2016). Several measures quantifying the effect of ambient conditions on cow performance have been explored (Igono et al., 1992; Bohmanova et al., 2007; Kendall et al., 2008), but few baselines have been documented for pre-weaned dairy calves in the Western US. For cows, THI has been recognized a good estimator for the magnitude of heat stress (West, 2003), where critical values have been stated at 76 units (Igono et al., 1992). Additionally, Bohmanova et al. (2007) established that the range of THI where milk yield declined was 72 to 77 units. For calves, a cut-off value of 77 THI units has been suggested to investigate the fluctuations of physiologic variables and health responses (Peña et al., 2016). In another report,

a THI ≥ 71 was significantly associated with greater disease incidence on Japanese black calves (Nabenishi and Yamazaki, 2016). In our analyses a cut-off 72 units was enough to evidence changes on behavior, respiratory rate, and rectal temperature.

As a positive effect provided by the ARC on calf health and performance would originate from the reduction in temperature and THI inside the hutch, multiple measuring points of temperature in the hutch have been proposed, including hutch inner wall, roof, and the use of radiation balls on the floor (Friend et al., 2014; Binion et al., 2014). In our analyses we included the effect of ambient THI category (<72; ≥72 units) at DP5 and DP6 on empty hutch temperature and THI, and on hutch wall temperature in hutches housing calves. Applying ARC lowered inner wall temperature only when ambient THI ≥72 units, which could be attributed to the sunlight reflection exerted by the ARC. Notably, covered empty hutches had higher overall average temperature and THI compared to control hutches. This contradictory finding could relate to the effect of ARC maintaining a higher inside temperature during the night and dawn periods, where temperatures were around 0ºC in some days for both study groups (Table 16). This evidence suggests that under conditions similar to this study, the aluminized insulation could be used as a strategy to maintain the inside hutch climate warmer during cooler periods of the day in areas where the ambient conditions fluctuate from low temperatures during night-dawn to high temperatures during sunlight periods.

During hot periods, calves usually seek shade and cooler surfaces to lay down (Roland et al., 2016). In our study, in days with high THI at DP5, a greater proportion of calves remained inside the control hutches compared to the covered hutches. However, this difference on behavior was lost when THI was low. Importantly, the installation of ARC covered the openings in the roof of the polyethylene hutches used in this study, which may have resulted in a significant reduction

in ventilation. This is in partial agreement with the findings presented by Carter et al. (2014) that, using a similar setting, reported that the behavior of the calves was influenced by the THI but not by the use of insulation.

The efficacy of ARC on improving hutch interior conditions during the hottest periods of the day (DP5 and DP6) differed for the two study period in this trial. The use ARC resulted in a significant reduction of empty hutch temperature and THI only during SG2. Nonetheless, this reduction in temperature and THI (0.5$^{o}$C and 0.5 units, respectively) did not have a clear effect on calf health and the physiological variables evaluated in our study.

**Conclusions**

The addition of aluminized reflective covers on polyethylene hutches was effective in reducing the inner hutch wall temperature in hutches housing calves during the hottest periods of the day. On the other hand, the use of covers maintained empty hutches warmer during the coldest periods of day. Our results suggest that the use of ARC did not generate a hutch microclimate that resulted in significant improvements on health and performance of pre-weaned dairy calves under these specific study settings.

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# APPENDIX

**Video 1.** Image sequence of the human active sorting (AS) behavior. Please notice how cows modified their orientation after seeing the sorter at the gate of the pen.

https://drive.google.com/open?id=1M0xN8DpcDKaXkbdRIG_JhF6cQiXY9tKC

**Video 2.** Image sequence of human passive sorting (PS) behavior. Please notice there was no active intervention to correct the direction of the cows. The observed behavior was produced by the presence of the sorter at the gate of the pen.

https://drive.google.com/open?id=12NSZPZIVaT7pBFTiU20UlzvfXCWIg0gr

**Video 3.** Image sequence of the gate sorting (GS) behavior. The only sorting element was the pen gate, opened in the same manner as in active sorting (AS) and passive sorting (PS).

https://drive.google.com/open?id=1ddPWnoINR9KT2JE0EHrXg-692YV9wRJn