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Diagnosis and prevalence of periparturient hypocalcemia and associated effects on milk production, reproductive performance and health of dairy cows in early lactation

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1   INTRODUCTION

The transition from late gestation to early lactation predisposes the dairy cow to an intense metabolic challenge. While the need for calcium is lowest when the cow is dried off and close to calving, the demand increases dramatically when the cow starts producing colostrum at parturition. Therefore, many dairy cows suffer either from clinical or subclinical hypocalcemia if mineral metabolism does not adapt adequately. Periparturient milk fever and subclinical hypocalcemia affect approximately every twentieth and every second multiparous cow, respectively (Reinhardt et al., 2011). To overcome the challenge of hypocalcemia, there are two mechanisms to provide accessible calcium in the cow. Low serum calcium concentration leads to a release of parathyroid hormone (**PTH**) from the parathyroid gland (Goff, 2008). This hormone activates vitamin D in order to not only absorb calcium from the gastro intestinal tract (**GIT**) passively via tight junctions, but also to activate a calcium pump leading to an active calcium transport from the lumen of the gut into the bloodstream. These ways to transport calcium are gene regulated and therefore take up to 48 hours to be activated (Martin-Tereso and Martens, 2014). The release of PTH also leads to mobilization of calcium from bone tissue. It is speculated, however, that this mechanism is time-consuming in multiparous cows because their mature skeleton has to recruit osteoclasts from progenitor cells in comparison to primiparous cows, in which bone modeling is still active (Goff, 2014). For these two reasons, calcium homeostasis postpartum is often critical, leading to a major risk period of 12 to 24 hours after parturition (Goff, 2008).

Preventive strategies have been developed, such as close-up diets restricted in calcium, vitamin D application one week before parturition, oral calcium supplementation around parturition, and feeding of anionic salts in the close-up diet in order to reduce prevalence of clinical and subclinical hypocalcemia. Recent studies have shown efficacy, especially for the concept of oral calcium supplementation (Oetzel and Miller, 2012) and feeding of anionic salts (Leno et al., 2017b). While vitamin D injections, diets restricted in calcium and anionic salts are often applied to all multiparous cows, additionally individual cows at high risk can be selected for oral calcium supplementation. Since most of the hypocalcemic animals are affected subclinically, there is an urgent need for a cow-side calcium test. In the absence of a validated test, however, the decision whether a cow should be selected for treatment, is often based on lactation number, and clinical appearance of the animal. It is well known that decreased skin and rectal temperature are indicative for hypocalcemia (Larsen et al., 2001). It has also been reported that periparturient hypocalcemia is associated with a low temperature of the ears (Guterbock, 2004; Radostits et al., 2007; Peek and Divers, 2008). Therefore, it is a common practice for herdsmen and veterinarians to evaluate calcium homeostasis of the cow by palpation of the ears. A further approach of calcium

supplementation is the treatment of subpopulations that are at high risk of developing hypocalcemia. Oetzel and Miller (2012) demonstrated that the application of calcium boluses can be efficient when treating fresh cows based on risk factors. In this study, lame cows were less susceptible to other diseases when they were treated with calcium orally. Cows with a high milk yield in the previous lactation produced 2.9 kg more milk at first dairy herd improvement association (**DHIA**) test when calcium was supplemented orally at parturition. Therefore, stochastic models were developed (McArt and Oetzel, 2015) to estimate the economic impact of oral calcium supplementation. In this study, not only treatment of targeted subpopulations (lame cows only, cows with high milk yield in previous lactation only, and treatment of both subpopulations), but also treatment of all multiparous cows was economically beneficial compared with no oral calcium supplementation.

The most cited reference for the prevalence of clinical and subclinical hypocalcemia is the study from Reinhardt et al. (2011). This study was based on 1,462 blood samples from 480 dairy herds from 21 states of the US and indicated that prevalence of milk fever was around 5 %. Approximately 25 % and 50 % of primiparous and multiparous cows, respectively, were affected by subclinical hypocalcemia, considering a threshold of 2.0 mmol/L. This study, however, was not specifically designed to estimate cow- and herd-level prevalence of hypocalcemia. The blood samples analyzed originated from the 2002 National Animal Health Monitoring System (**NAHMS**) dairy study. In the meantime, fresh cow management has improved and preventive strategies have been implemented on dairy farms in order to avoid hypocalcemia. In 2002, the proportion of heifers and multiparous cows fed with anionic salts in the close-up diet was 14.3 % and 19.1 %, respectively (USDA, 2002). In a more recent NAHMS report 20.7 % and 27.6 % of heifers and multiparous cows had been fed with anionic salts, respectively (USDA, 2014). Furthermore, it is questionable if the data from Reinhardt et al. (2011) are applicable to the European dairy industry and whether prevalence of clinical and subclinical hypocalcemia should therefore be re-evaluated under local and current farming conditions.

In the recent literature, there is evidence for detrimental effects of hypocalcemia on early lactation milk yield, animal health, and reproductive performance. In part, a higher susceptibility to postpartum diseases can be explained by an immunosuppressive effect of hypocalcemia. While there is an intracellular calcium release response of peripheral mononuclear cells to an immune cell activation signal, Kimura et al. (2006) reported that intracellular calcium stores were decreased in peripheral mononuclear cells in a state of hypocalcemia. As a result, the intracellular calcium release was blunted, leading to an inadequate immune response. Martinez et al. (2014) were able to show that the ability of neutrophils to undergo phagocytosis and oxidative burst was impaired in cows suffering from

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hypocalcemia. In addition, the number of neutrophils was reduced in hypocalcemic cows compared with normocalcemic cows. Epidemiologic studies proved that hypocalcemia around parturition was associated with a higher susceptibility to infectious diseases such as metritis (Martinez et al., 2012; Ribeiro et al., 2013; Rodriguez et al., 2017; Wilhelm et al., 2017) and mastitis (Curtis et al., 1983). Since hypocalcemia affects skeletal and smooth muscle contraction, ultimately resulting in recumbency, the risk to develop a displaced abomasum was also higher in cows with a low serum calcium concentration (Chapinal et al., 2011; Seifi et al., 2011; Rodriguez et al., 2017). In addition, recent studies reported also a reduction of rumen contractions and dry matter intake (Martinez et al., 2014) and a higher loss of body weight postpartum (Caixeta et al., 2015) in cows affected by hypocalcemia. Furthermore, hypocalcemic animals had a greater risk of high NEFA postpartum and were more prone to develop ketosis (Ribeiro et al., 2013; Rodriguez et al., 2017). Comparing these studies, it is obvious, however, that different risk periods from 24 hours up to three weeks postpartum were considered. Long risk periods must be interpreted with caution, as it is also plausible that reduced feed intake before clinical signs of disease can affect serum calcium concentration (Pinedo et al., 2017).

Several studies also tried to elucidate the impact of periparturient hypocalcemia on reproductive performance. Chapinal et al. (2012b) showed that cows with a serum calcium concentration above 2.3 mmol/L one week before parturition or a serum calcium concentration above 2.2 mmol/L one week postpartum had greater odds of pregnancy at first service (odds ratio (**OR**) = 1.5 and 1.3, respectively). Further evidence exists that the proportion of cows being cyclic by 60 days in milk (**DIM**) was greater when cows were normocalcemic (threshold = 2.14 mmol/L; OR = 0.32; Rodriguez et al., 2017).

Information about the impact of hypocalcemia on early lactation milk yield is controversial. While there is one study that found decreased milk yield up to 120 DIM when cows were suffering from hypocalcemia (Chapinal et al., 2012b), another study found no effect of hypocalcemia on milk production (Martinez et al., 2012). Some authors even reported higher milk yield until the third DHIA (Gild et al., 2015). Therefore, further research is required to answer the question whether periparturient hypocalcemia has an impact on milk production.

The overall objectives of this thesis were (1) to evaluate ear skin temperature as a cow-side test to predict serum calcium concentration, (2) to determine the prevalence of clinical and subclinical hypocalcemia in German dairy herds, and (3) to evaluate the impact of hypocalcemia on early lactation milk yield, reproductive performance, and culling risk in early lactation.

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Introduction

Results of publication I and publication II have been published in the Journal of Dairy Science (Impact Factor: 2.474). Data from the additional unpublished work have been formatted according to the guidelines of the same journal and will be submitted shortly.

2 PUBLICATION I

Evaluation of ear skin temperature as a cow-side test to predict postpartum calcium status in dairy cows

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2.1   Abstract

Subclinical hypocalcemia is considered a gateway disease that increases susceptibility to other metabolic and infectious diseases in transition dairy cows. In the absence of a cow-side test, however, it is difficult to identify hypocalcemic cows. The objective of this study was to evaluate ear skin temperature as a diagnostic predictor for serum calcium concentration. A cross-sectional study was conducted on seven commercial dairy farms, involving 251 cows 0 to 48 hours after calving. Skin temperature of the ears (**STEar**) was scored manually by palpation of both ears. An infrared thermometer was used to measure ear temperature, skin temperature on the coxal tuber (**STCox**), and ambient temperature (**AT**). Rectal temperature (**RT**) was measured with a digital thermometer. A blood sample was drawn to determine serum calcium concentration. Hypocalcemia was defined as serum calcium below 2.0 mmol/L irrespective of clinical symptoms. Serum calcium concentration below 2.0 mmol/L in connection with clinical symptoms was defined as clinical milk fever, whereas serum calcium concentration below 2.0 mmol/L in absence of clinical symptoms was defined as subclinical hypocalcemia. A multivariate analysis using the GENLINMIXED procedure and a ROC-curve analysis was performed to evaluate whether serum calcium concentration can be predicted by measuring ear temperature and other temperature estimates. Prevalence of hypocalcemia was 3.3 %, 27.3 %, 32.8 %, and 69.6 % for cows in first, second, third, and ≥ fourth lactation, respectively. None of the cows in first and second lactation were suffering from clinical milk fever. Prevalence of clinical milk fever was 6.0 % and 20.3 % for third and ≥ fourth lactation cows, respectively. There was an association between serum calcium concentration and temperature of the ears. A decrease in ear temperature of 0.39 °C (95 % CI 0.25 – 0.54; *P* = 0.001) was associated with a decrease of 0.1 mmol/L in serum calcium concentration. Ambient temperature, however, was a major confounder for ear temperature. When there was an increase in ambient temperature of 1 °C, STEar rose by 0.78 °C (95 % CI 0.67 – 0.90; *P* = 0.001). Hypothermia was more pronounced in clinical milk fever (median 21.8 °C; IQR 14.7 °C – 27.0 °C) compared to subclinical hypocalcemia (median 27.6 °C, IQR 22.1 °C – 30.8 °C). All temperature estimates had only accurate test characteristics based on their AUC for prediction of subclinical hypocalcemia (AUC for STEar, STCox, and RT were 0.641, 0.668 and 0.606, respectively) when cows with clinical milk fever were excluded. Although an association between ear temperature and serum calcium concentration could be shown, ear temperature cannot be recommended for diagnosis of subclinical hypocalcemia.

2.2   Key words

subclinical hypocalcemia, milk fever, ear skin temperature

2.3   Introduction

Hypocalcemia is a potentially life-threatening metabolic disorder of dairy cows that predisposes the animal to various other metabolic and infectious disorders (Goff, 2008). It can be clinical or subclinical. Typically, the nadir in blood calcium concentration occurs between 12 and 24 h after calving and only blood samples obtained at this time can reveal the extent of hypocalcemia experienced by a dairy herd (Kimura et al., 2006; Goff, 2008). Subclinical hypocalcemia is defined as a concentration of calcium in serum less than 2.0 mmol/L and affects approximately 50 % of second and greater lactation and up to 25 % of first lactation animals (Reinhardt et al., 2011).

Hypocalcemia around calving is associated with reduced milk yield, increased risk for clinical diseases (e.g., displaced abomasum, metritis) and increased culling risk in early lactation (Chapinal et al., 2011; Seifi et al., 2011; Chapinal et al., 2012a; Chapinal et al., 2012b; Roberts et al., 2012). Subclinical hypocalcemia is more costly than clinical milk fever because it affects a higher percentage of cows in the herd.

Supplementation with oral calcium formulations around calving in cows with subclinical hypocalcemia can reduce the risk of postpartum problems and increase the milk yield (Oetzel, 2013). There is no comprehensively validated cow-side blood calcium test available to identify cows with subclinical hypocalcemia except for a hand-held photometric test for calcium in blood validated with only 20 samples described in a preliminary report (Bootz et al., 2014). Known risk factors of hypocalcemia (e.g., parity, high milk yield in previous lactation, lameness) have been recommended as a way of identifying subpopulations of cows in which oral calcium supplementation would be beneficial (Oetzel and Miller, 2012), but this approach may lead to the unnecessary treatment of cows that have risk factors but not hypocalcemia (i.e., false-positive treatment decisions). More importantly, cows that have hypocalcemia but do not show risk factors (false negatives) will be missed.

It is well known that decreased temperature of the ear (Guterbock, 2004; Radostits et al., 2007, Peek and Divers, 2008) and skin (Larsen et al., 2001) are clinical symptoms indicative of hypocalcemia in periparturient dairy cows. Therefore, it is a common practice for veterinarians and herdsmen to use the ear temperature determined by manual palpation as an estimate for the presence or absence of milk fever (i.e., calcium status) of a periparturient cow. To our knowledge, however, temperature of the ear skin has never been validated as a potential predictor of calcium status.

Infrared thermography allows to measure surface skin temperature noninvasively and does not lead to any radiation exposure (Eddy et al., 2001; Schaefer et al., 2004). These characteristics lead to an emerging interest in human and veterinarian medicine. The technique has previously been used in cows to assess skin temperature of the udder and hooves in order to identify subclinical mastitis (Colak et al., 2008) and laminitis (Nikkhah et al. (2005), respectively.

Measuring the skin temperature of the ears would be a fast and non-invasive tool to assess the calcium status of the cow. Therefore, the objective of our study was to evaluate diagnostic performance of ear skin temperature to identify cows with hypocalcemia.

2.4   Materials and methods

The experimental procedures reported herein were conducted with the approval of the Institutional Animal Care and Use Committee of the Freie Universität Berlin. Cows were managed according to the guidelines set by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products (Hellmann & Radeloff, 2000)

2.4.1   Cows, housing, and feeding

A cross-sectional clinical study was conducted from January 2015 to August 2015. A convenience sample of seven commercial dairy farms were recruited within a 165 km radius of a veterinary practice located in Brandenburg, Germany. Average herd size was 717 (range 270 – 1,425). The average milk production (305 d ECM, 4.0 % fat, 3.4 % protein) was 8,766 kg (range 7,470 kg – 9,634 kg). All cows were Holstein cows except for herd 3 with Jersey cows as the dominant breed. Lactating cows were milked twice daily and for all cows, TMR was delivered once daily and pushed up multiple times per day. The TMR from close-up and fresh cows was formulated to meet or exceed minimum nutritional requirements for high-producing dairy cows (NRC, 2001). None of the herds used anionic salts as a prevention strategy for milk fever. Only herd 1 implemented a blanket treatment for third or greater parity cows with a fat-coated bolus directly after parturition that consisted of 43 g of calcium (Bovikalc, Boeringer Ingelheim Pharma GmbH & Co. KG, Ingelheim am Rhein, Germany).

Milking cows in all herds were housed in freestalls. In herds 1, 2, and 4, dry cows were housed in freestall barns with slatted floors and beds equipped with straw (herd 4) or wood shavings (1, 2). Dry cows in herd 3, 5, 6 and 7 were housed on deep straw bedding.

Overall, 251 animals were enrolled. Inclusion criteria was calving within the last 48 hours at a given day (i.e., during routine herd health visit).

2.4.2 Experimental procedures

Cows were restrained in headlocks during all experimental procedures. All cows enrolled in this study were examined 0 to 48 hours after calving by one veterinarian according to a written standard operating procedure (**SOP**). Briefly, skin temperature of the ears (**STEar**) was scored manually by palpation of both ears on a 3-point scale (1 = 5-15 °C; 2 = 16-25 °C; 3 = 26-35 °C). For measuring STEar (Figure 1), an infrared thermometer (**IRT**; FLUKE 568 IR Thermometer, Fluke Deutschland GmbH, Glottertal, Germany) was used on the front and rear side of each ear. The device featured a range from -40 to 900 °C with a resolution of 0.1 °C, a spectral range from 8 µm to 14 µm and a sensitivity of ± 1 % or ± 1 °C. We used an emissivity of 0.98 as previously described for the skin of calves (Hoffmann et al. 2012), other mammals (Kastberger and Stachel 2003) and human skin (Wolfe and Zissis 1985).

Temperature was detected with a distance of approximately 1 m and a measured area of 2 cm in diameter. Skin temperature on the coxal tuber (**STCox**) was measured the same way as described for STEar. Rectal temperature (**RT**) was measured using a commercial thermometer (Veterinär-Thermometer SC 12, SCALA Electronic GmbH, Stahnsdorf, Germany).

Temperature of the feeding alley was recorded using the IRT in order to obtain an estimate of the ambient temperature (**AT**).

2.4.3 Blood sampling and laboratory analyses

Blood samples were taken immediately after evaluation of the skin temperature from the coccygeal vessels using vacutainer systems (Vacuette 8 ml Z Serum Beads Clot Activator, Greiner Bio-One GmbH, Kremsmünster, Austria). Samples were kept at room temperature and allowed to clot. Within 5 h of blood collection, samples were centrifuged to harvest serum, which was frozen at -20 °C. Analysis of blood samples was carried out by a commercial laboratory (Synlab Services GmbH, Augsburg, Germany). Total serum calcium concentration was analyzed using photometry (AU680, Beckman Coulter, Krefeld, Germany). The inter- and intraassay coefficients of variation were 1.6 % and 0.7 %, respectively.

2.4.4   Statistical analyses

The study was carried out as an observational experiment. Animals were enrolled by convenience when a veterinarian visited the farm on a given day and an animal met the inclusion criteria of being within 48 h after parturition.

Individual cow data were transferred to Microsoft Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany). Statistical analyses were performed using SPSS for Windows (version 22.0, SPSS Inc., IBM, Ehningen, Germany). The individual cow was the experimental unit in all analyses.

Univariate analyses were used to determine the association between STEar, STCox or RT and calcium status. Calcium status was defined according to the serum calcium concentration and the clinical appearance of the animal. Normocalcemia was defined as serum calcium concentration greater or equal to 2.0 mmol/L. Cows not affected clinically with a serum calcium concentration below 2.0 mmol/L were characterized as subclinical hypocalcemic animals. Recumbent cows with a serum calcium concentration below 2.0 mmol/L were defined as cows suffering from clinical milk fever.

The univariate analyses included STEar, STCox or RT as dependent variable and calcium status as independent variable. For evaluation of the association between serum calcium concentration and STEar, STCox or RT, we used the GENLINMIXED procedure of SPSS. Herd was considered as a random effect. According to the model-building strategies described by Dohoo et al. (2009), each parameter considered for the mixed model should be separately analyzed in a univariate model, including the parameter as a fixed factor (i.e., categorical parameter) or covariate (i.e., continuous parameter). Only parameters resulting in univariate models with $P \leq 0.20$ should be included in the final mixed model. The initial model contained the following explanatory variables as fixed effects: parity (1, 2, 3, greater or equal to 4), breed (Holstein vs. Jersey), time after parturition (continuous; 0 to 48 h), oral calcium supplementation (yes vs. no), STEar (continuous), RT (continuous), STCox (continuous).

Selection of the model that best fit the data was performed by finding the model with the lowest value for the Akaike information criterion (AIC) using a backward elimination procedure that removed all variables with $P > 0.10$ from the model.

Additionally, we evaluated the factors influencing STEar using a GENLINMIXED procedure. Therefore, STEar was the independent variable. Model building and selection of the model that best fit the data was performed as described above. The initial model contained the following explanatory variables as fixed effects: lactation group (1 to 4), breed (Holstein vs.

Jersey), time after parturition (continuous; 0 to 48 h), blood calcium (continuous), oral calcium supplementation (yes vs. no), AT (continuous), RT (continuous).

In order to define reference criteria to identify cows with subclinical hypocalcemia based on their skin (i.e., STEar, STCox) or rectal temperature, the receiver operating characteristic (ROC) analysis was used. The continuous variable was either skin or rectal temperature and the classification variable was the calcium status. The ROC curves analyses sensitivity versus 100-specificity. For differentiation between normocalcemia and hypocalcemia, four different thresholds were used, which were associated with negative health or production outcomes (Chapinal et al., 2011, 2012a,b; Seifi et al., 2011; Martinez et al. 2012; Roberts et al. 2012). Sensitivity is the proportion of cows diagnosed hypocalcemic that had serum calcium concentrations below the threshold; specificity is the proportion of cows diagnosed normocalcemic that had blood calcium above the threshold (Greiner et al., 2000). The point on the ROC curve with the highest combined sensitivity and specificity was considered the critical threshold. Interpretation of this critical threshold was based on the area under the curve (**AUC**) according to Swets (1998) as non-informative (AUC = 0.5), accurate (0.5 < AUC ≤ 0.7), very accurate (0.7 < AUC ≤ 0.9), highly accurate (0.9 < AUC < 1), and perfect (AUC = 1).

A significant difference between the levels of a classification variable was declared when $P < 0.05$, whereas differences between $P ≥ 0.05$ and $P ≤ 0.10$ were considered a statistical tendency.

2.5   Results

Data of 251 animals were available for final analyses. Sixty of those were primiparous cows (23.9 %); 55 (21.9 %), 67 (26.7 %), and 69 (27.5 %) cows were in second, third, and ≥ fourth lactation, respectively.

Mean value for the interval between calving and investigation of the ear temperature was 19.96 hours (SD 13.91). Overall, the prevalence of subclinical hypocalcemia and clinical milk fever was 27.4 % (69/251) and 7.2 % (18/251), respectively. The prevalence of hypocalcemia increased with lactation number. Prevalence was 3.3 % (2/60), 27.3 % (15/55), 32.8 % (22/67), and 69.6 % (48/69) for cows in first, second, third and fourth or greater lactation, respectively.

None of the cows in first and second lactation were suffering from clinical milk fever. Prevalence of clinical milk fever was 6.0 % (4/67) and 20.3 % (14/69) for third and ≥ fourth lactation cows, respectively.

As indicated by the multivariate analyses, there was a positive association between each of the three animal temperature measures (STEar, STCox, RT) and serum calcium concentration (Table 1 to 3). Hypothermia was more pronounced in clinical milk fever compared to subclinical hypocalcemia for STEar (Figure 2, Panel A), STCox (Figure 2, Panel B), and RT (Figure 2, Panel C), respectively. Ear skin temperature, STCox, and RT were analyzed with ROC curves to determine the critical thresholds (combined highest sensitivity and specificity) in order to distinguish between normocalcemia and subclinical hypocalcemia, excluding animals with clinical milk fever (Table 4). The AUC for the differentiation between normocalcemia and subclinical hypocalcemia based on STEar, STCox, and rectal temperature were 0.641, 0.668, and 0.606, respectively (P = 0.001).

We identified AT as a potential confounder for STEar (Table 5). However, there was still an association between serum calcium concentration and STEar when we considered AT in the same model.

There was a high correlation between STEar and manual ear score (r = 0.76; P = 0.001). Additionally, we tested the association between the manual ear score and serum calcium concentration. Multivariate analysis indicated an association between manual ear score and serum calcium concentration. Compared to cows with ear score one, estimated serum calcium concentration was 0.346 mmol/L (95 % CI 0.193 – 0.498; P = 0.001) and 0.432 mmol/L (95 % CI 0.291 – 0.573; P = 0.001) higher for cows with ears score two and three, respectively.

Table 1. Association between serum calcium concentration (mmol/L) and ear skin temperature (STEar) based on multivariate analysis.

Variable[1]	Estimate	SE[2]	95 % Confidence Interval		P
	mmol/L		Lower CI	Upper CI	
Intercept	1.867	0.112	1.646	2.087	0.001
Lactation group					
Lactation 1	Referent				
Lactation 2	-0.047	0.063	-0.171	0.076	0.450
Lactation 3	-0.199	0.059	-0.315	-0.082	0.001
Lactation 4+	-0.483	0.062	-0.606	-0.360	0.001
Breed					
Holstein	Referent				
Jersey	-0.113	0.052	-0.216	-0.010	0.032
Skin temperature of the ear, °C	0.012	0.003	0.005	0.019	0.001

[1] Model adjusted for the random effect of herd.

[2] SE = Standard error of the estimate.

Table 2. Association between serum calcium concentration (mmol/L) and skin temperature on the coxal tuber (STCox) based on multivariate analysis.

Variable[1]	Estimate	SE[2]	95 % Confidence Interval		*P*
	mmol/L		Lower CI	Upper CI	
Intercept	1.379	0.312	0.764	1.994	0.001
Lactation group					
Lactation 1	Referent				
Lactation 2	-0.042	0.062	-0.164	0.079	0.495
Lactation 3	-0.200	0.058	-0.315	-0.085	0.001
Lactation 4+	-0.479	0.061	-0.599	-0.358	0.001
Breed					
Holstein	Referent				
Jersey	-0.088	0.052	-0.191	0.015	0.094
Skin temperature, of the coxal tuber, °C	0.027	0.006	0.015	0.038	0.001

[1] Model adjusted for the random effect of herd.

[2] SE = Standard error of the estimate.

Table 3. Association between serum calcium concentration (mmol/L) and rectal temperature (RT) based on multivariate analysis.

Variable[1]	Estimate	SE[2]	95 % Confidence Interval		P
	mmol/L		Lower CI	Upper CI	
Intercept	-3.157	1.559	-6.229	-0.084	0.044
Lactation group					
Lactation 1	Referent				
Lactation 2	-0.031	0.063	-0.156	0.094	0.621
Lactation 3	-0.164	0.060	-0.282	-0.045	0.007
Lactation 4+	-0.460	0.064	-0.586	-0.333	0.001
Breed					
Holstein	Referent				
Jersey	-0.081	0.054	-0.189	0.026	0.137
Rectal temperature, °C	0.137	0.040	0.059	0.215	0.001

[1] Model adjusted for the random effect of herd.

[2] SE = Standard error of the estimate.

Table 4. Critical thresholds for ear skin temperature (STEar), skin temperature on the coxal tuber (STCox) and rectal temperature (RT) to differentiate between normocalcemia and subclinical hypocalcemia based on receiver operating characteristic analyses and considering four different calcium thresholds to define subclinical hypocalcemia.

Ca Threshold	Prevalence, %	Temperature variable, °C	Threshold, °C	Se[1]	Sp[2]	AUC	*P*
2.0 mmol/L	29.6	STEar	27.0	49.3	73.8	0.641	0.001
		STCox	30.0	52.2	78.7	0.668	0.001
		RT	39.0	75.4	42.7	0.606	0.009
2.1 mmol/L	45.1	STEar	27.9	47.6	71.8	0.615	0.001
		STCox	30.9	54.3	63.3	0.597	0.010
		RT	38.5	28.6	85.2	0.576	0.046
2.2 mmol/L	65.2	STEar	29.4	53.3	66.7	0.600	0.007
		STCox	30.8	50.0	70.4	0.600	0.009
		RT	39.3	21.7	90.1	0.536	0.350
2.3 mmol/L	84.5	STEar	28.3	42.1	77.8	0.607	0.026
		STCox	30.5	40.6	77.8	0.580	0.090
		RT	39.1	31.0	83.3	0.557	0.224

[1] Se = Sensitivity

[2] Sp = Specificity

Table 5. Association between ear skin temperature (STEar) and serum calcium concentration considering ambient temperature (AT).

Variable[1]	Estimate	SE[2]	95 % Confidence Interval		P
	°C		Lower CI	Upper CI	
Intercept	6.683	1.942	2.856	10.509	0.001
Ambient temperature, °C	0.783	0.059	0.667	0.899	0.001
Calcium, mmol/L	3.918	0.734	2.472	5.364	0.001

[1] Model adjusted for the random effect of herd.
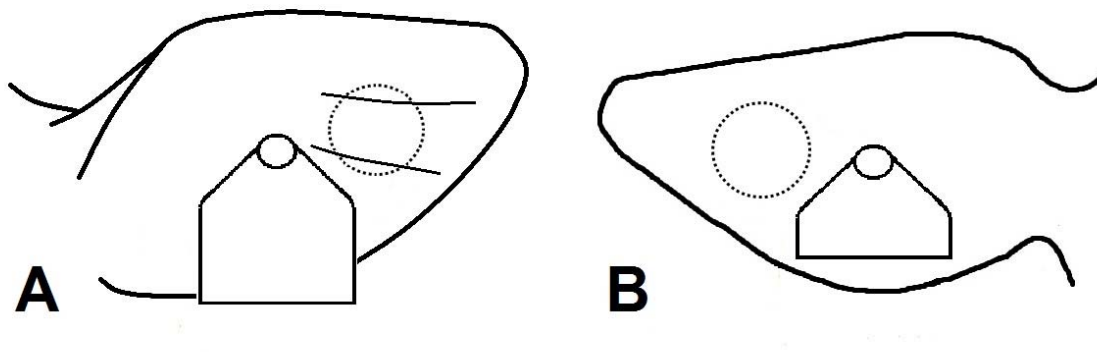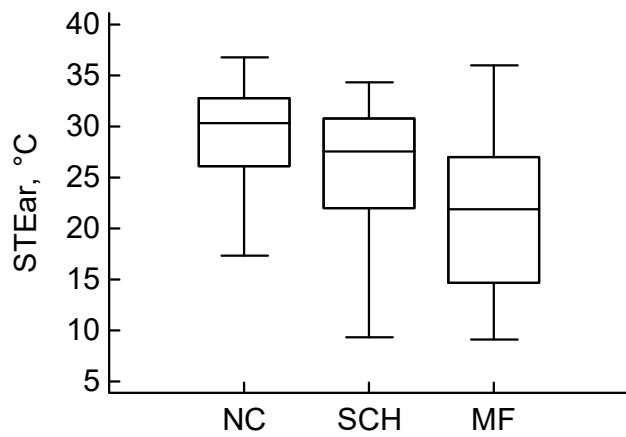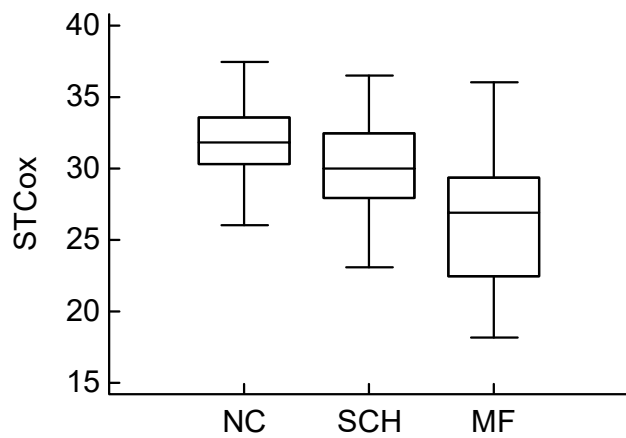
[2] SE = Standard error of the estimate.

Figure 1. Schematic presentation of the measuring points for the infrared thermometer on the front (A) and rear side (B) of the ear.
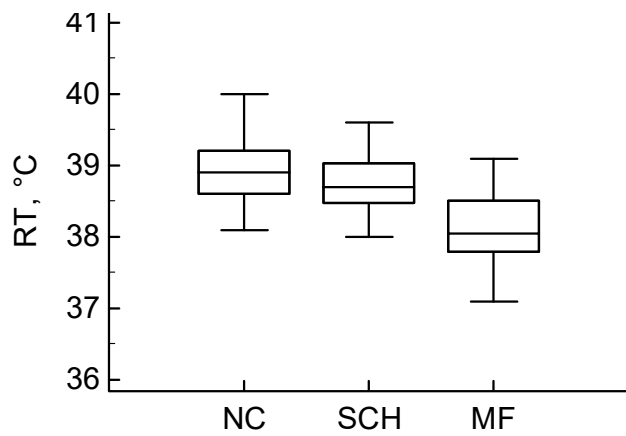
Figure 2. Association between ear skin temperature (STEar; panel A), skin temperature on the coxal tuber (STCox; panel B), rectal temperature (RT; panel C) and calcium status [normocalcemia (NC) n = 164; subclinical hypocalcemia (SCH) n = 69; milk fever (MF) n = 18]. The central box represents the interquartile range from the first to third quartile. A segment inside the box shows the highest and lowest case within 1.5 times the interquartile range, respectively.

2.6   Discussion

It is well known that cows suffering from clinical milk fever have cold extremities (Guterbock, 2004, Radositits, 2007). Therefore, many farmers and practitioners implement manual palpation of the ears in fresh cow protocols, in order to evaluate the calcium status and provide calcium supplementation based on this sensorial but subjective observation.

The objective of this study was to evaluate whether this procedure might be a useful predictor to identify not only cows with clinical milk fever, but also cows that were subclinically hypocalcemic. Hypocalcemia was more prevalent in older cows in our study, which is in agreement with previous results. Reinhardt et al. (2011) showed that the serum calcium concentration declined significantly with increasing lactation number. In the same experiment, the concentration of 1,25-dihydroxyvitamin D (**1,25(OH$_2$)D**) increased from first to third lactation, but plateaued beyond third lactation which might indicate that the endocrine adaptation is inadequate in these cows.

Horst et al. (1990) showed in rats that the concentration of 1,25(OH$_2$)D receptors in the intestine and the bone was declining significantly with increasing age. Goff (2014) assumed for dairy cows that the inability to maintain calcium homeostasis was due to the mature skeleton and reduced response to parathyroid hormone (**PTH**) in older animals. Bone remodeling was reduced and active osteoclasts and osteoblasts are scarce. It was also demonstrated that PTH receptors in the kidneys are downregulated with age in rats (Hanai et al. 1990). This might also occur in osteoclasts and osteoblasts of cows being another reason for maladaptation of the mineral metabolism after parturition (Goff, 2014).

Wittek et al. (2011) assumed that postpartum calcium homeostasis and the ability to mobilize calcium might be dependent on immunological processes. They observed a difference in the expression of different immunological profiles of cytokines and chemokines from first lactation cows compared to cows in higher lactation. However, further research regarding the role of the immune system in the etiology of hypocalcemia is indicated.

Using multivariate analysis, we compared serum calcium concentrations with the temperatures measured on the surface of the ears. It was obvious that there is only a moderate relationship between serum calcium concentration and STEar. Hypothermia seems to be more pronounced in cows suffering from clinical milk fever. However, a major confounder was AT. This finding is consistent with a previous study describing that the temperature of hooves in horses and cows depends markedly on AT (Gloster et al., 2011). It was not possible to sample healthy cows under the same environmental conditions as a control group as a valid cow-side test is not available. Such a comparison might have improved the results of our study.

Cows suffering from hypocalcemia had also a lower surface temperature of the skin on the coxal tubers (Radostits, 2007) as well as a lower rectal temperature (Larsen et al., 2001).

Maladaptation of the mineral metabolism to periparturient calcium loss via colostrum and milk production leads to calcium deficiency and diminished muscle contraction, finally resulting in recumbency (Goff, 2004). One explanation for the reduced skin and core temperature observed in our study might be this decreased muscle contraction causing lower thermal energy production. Furthermore, dry matter intake is reduced in periparturient cows (Grummer et al., 2004) which might decrease the heat of fermentation and therefore core temperature.

Another reason for a decreased body temperature is the drop of serum progesterone concentration around calving (Suthar et al., 2012) which can be used for the prediction of birth in dairy cows (Burfeind et al., 2011).

As stated by Lima and Bakker (2005), sensorial assessment of surface temperature in humans can be used to diagnose reduced peripheral perfusion in shock like situations. Due to a decrease in cardiac output, clinical milk fever might also go along with reduced blood circulation of the extremities and therefore result in colder ears.

The objective of this study was to evaluate whether STEar can be used to identify cows suffering from hypocalcemia. In conclusion, hypothermia was a poor cow-side predictor for diagnosis of subclinical hypocalcemia as evidenced by the results from the ROC curves analyses. Hypothermia was more pronounced in cows suffering from clinical milk fever. Therefore, measuring ear temperature has a certain but limited value to reflect calcium status. Overall, it cannot be recommended as a diagnostic test for subclinical hypocalcemia. In the absence of a validated cow-side blood test for hypocalcemia, further development and research approaches are warranted to improve the current concept of using risk factors as decision criteria for individual calcium supplementation in fresh cows (Oetzel & Miller, 2012).

2.7   Acknowledgements

2.8   References

Bootz, F., M. Hostens, and B. Van Ranst. 2014. A new portable blood/urine cow side test for NEFA, Calcium, Lactate and Magnesium. Page 94 in XXVIII World Buiatrics Congress. Cairns 2014, Australian Veterinary Association, New South Wales, Saint Leonards.

Burfeind, O., V. S. Suthar, R. Voigtsberger, S. Bonk, and W. Heuwieser. 2011. Validity of prepartum changes in vaginal and rectal temperature to predict calving in dairy cows. J. Dairy Sci. 94:5053-5061.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. J. Dairy Sci. 94:4897-4903.

Chapinal, N., M. E. Carson, S. J. LeBlanc, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012a. The association of serum metabolites in the transition period with milk production and early-lactation reproductive performance. J. Dairy Sci. 95:1301-1309.

Chapinal, N., S. J. Leblanc, M. E. Carson, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012b. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. J. Dairy Sci. 95:5676-5682.

Colak A., B. Polat, Z. Okumus, M. Kaya, L. E. Yanmaz and A. Hayirli. 2008. Short communication: Early detection of mastitis using infrared thermography an dairy cows. J. Dariy Sci. 91:4244-4248

Dohoo, I. R., S. W. Martin, and H. Stryhn. 2009. Veterinary Epidemiologic Research (2nd). University of Prince Edward Island, Charlottetown, PEI, Canada.

Eddy, A. L., L. M. van Hoogmoed, and J. R. Snyder. 2001. Review: The role of thermography in the management of equine lameness. Vet. J. 162:172-181.

Gloster, J., K. Ebert, S. Gubbins, J. Bashiruddin, and D. J. Paton. 2011. Normal variation in thermal radiated temperature in cattle: implications for foot-and-mouth disease detection. BMC Vet. Res. 7:73.

Goff, J. P. 2004. Macromineral disorders of the transition cow. Vet. Clin. North Am. Food Anim. Pract. 20:471-494, V.

Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Vet. J. 176:50-57.

Goff, J. P. 2014. Calcium and magnesium disorders. Vet. Clin. North Am. Food Anim. Pract. 30:359-381, VI.

Greiner, M., D. Pfeiffer, R. D. Smith. 2000. Principles and practical application of the receiver operating characteristic analysis for diagnostic tests. Prev. Vet. Med. 45:23-41.

Grummer, R. R., D. G. Mashek, and A. Hayirli. 2004. Dry matter intake and energy balance in the transition period. Vet. Clin. North Am. Food Anim. Pract. 20:447-470.

Guterbock, W. M. 2004. Diagnosis and treatment programs for fresh cows. Vet. Clin. North Am. Food Anim. Pract. 20:605-626, VII.

Hanai, H., D. P. Brennan, L. Cheng, M. E. Goldman, M. Chorev, M. A. Levine, B. Sacktor, and C. T. Liang. 1990. Downregulation of parathyroid hormone receptors in renal membranes from aged rats. Am. J. Physiol. 259:444-450.

Hellmann, K. and I. Radeloff. 2000. International Cooperation on Harmonisation of Technical Requirements of Veterinary Medicinal Products (VICH). Brussels/Belgium. Accessed May 3, 2016.
http://www.fda.org/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/Guidance forIndustry/UCM052417.pdf.

Hoffmann, G., M. Schmidt, C. Ammon, S. Rose-Meierhofer, O. Burfeind, W. Heuwieser, and W. Berg. 2013. Monitoring the body temperature of cows and calves using video recordings from an infrared thermography camera. Vet. Res. Commun. 37:91-99.

Horst, R. L., J. P. Goff, and T. A. Reinhardt. 1990. Advancing age results in reduction of intestinal and bone 1,25-dihydroxyvitamin D receptor. Endocrinology 126:1053-1057.

Kastberger, G. and R. Stachl. 2003. Infrared imaging technology and biological applications. Behav. Res. Methods Instrum. Comput. 35:429-439.

Kimura, K., T. A. Reinhardt, and J. P. Goff. 2006. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. J. Dairy Sci. 89:2588-2595.

Larsen, T., G. Moller, and R. Bellio. 2001. Evaluation of clinical and clinical chemical parameters in periparturient cows. J. Dairy Sci. 84:1749-1758.

Lima, A. and J. Bakker. 2005. Noninvasive monitoring of peripheral perfusion. Intensive Care Med. 31:1316-1326.

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvao and J. E. P. Santos. 2012. Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk in developing uterine disease. J. Dairy Sci. 95:7158-7172.

Nikkah, A., J. C. Plaizier, M.S. Einarson, R. J. Berry, S. L. Scott, and A. D. Kennedy. 2005. Infrared thermography and visual examination in hooves of dairy cows in two stages of lactation. J. Dairy Sci. 88:2749-2753.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th ed. Natl. Acad. of Sci., Washington, DC.

Oetzel, G. R. 2013. Oral calcium supplementation in peripartum dairy cows. Vet. Clin. North Am. Food Anim. Pract. 29:447-455.

Oetzel, G. R. and B. E. Miller. 2012. Effect of oral calcium bolus supplementation on early-lactation health and milk yield in commercial dairy herds. J. Dairy Sci. 95:7051-7065.

Peek, S. and T. Divers. 2008. Metabolic Diseases. Pages 590-605 in Rebhuns Diseases of Dairy Cattle. T. Divers and S. Peek, ed. Saunders Elsevier, St Louis, MO.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Vet. J. 188:122-124.

Radostits, O. M., K. W. Hinchcliff, and P. D. Constable. 2007. Veterinary Medicine. A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats. 10th ed. W.B. Saunders Company Ltd. London, UK.

Roberts, T., N. Chapinal, S. J. Leblanc, D. F. Kelton, J. Dubuc, and T. F. Duffield. 2012. Metabolic parameters in transition cows as indicators for early-lactation culling risk. J. Dairy Sci. 95:3057-3063.

Schaefer, A. L., N. Cook, S. V. Tessaro, D. Deregt, G. Desroches, P. L. Dubeski, A. K. W. Tong, and D. L. Godson. 2004. Early detection and prediction of infection usind infrared thermography. Can. J. Anim. Sci. 84:73-80.

Seifi, H. A., S. J. Leblanc, K. E. Leslie, and T. F. Duffield. 2011. Metabolic predictors of post-partum disease and culling risk in dairy cattle. Vet. J. 188:216-220.

Suthar, V. S., O. Burfeind, S. Bonk, A. J. Dhami, and W. Heuwieser. 2012. Endogenous and exogenous progesterone influence body temperature in dairy cows. J. Dairy Sci. 95:2381-2389.

Swets, J. A. 1988. Measuring the accuracy of diagnostic systems. Science 240:1285-1293.

Wittek, T., Jonnson, N. 2011. Gebärparese – Warum erkranken ältere Kühe? – Eine Hypothese. Page 77 in Prophylaxe von Herden- bzw. Produktionskrankheiten. Leipzig, Germany

Wolfe, W. L., G. J. Zissis. 1985. The infrared handbook, revised edn. Office of Naval Research, Department of Navy, Washington, D.C. General Dynamics, Virginia.

3   Publication II

Hypocalcemia – Cow-level prevalence and preventive strategies in German dairy herds

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3.1 Abstract

Hypocalcemia around calving is considered a gateway disease that can lead to health disorders and decreased milk production. The objective of this cross-sectional study was to evaluate the prevalence of clinical and subclinical hypocalcemia 0 to 48 h after calving. Blood samples were drawn from 12 animals of each dairy farm (n=115) and analyzed for serum calcium, magnesium, and phosphorus concentration. Cows not affected clinically but with a serum calcium concentration below 2.0 mmol/L were characterized as subclinical hypocalcemic animals. Recumbent cows with a serum calcium concentration below 2.0 mmol/L were defined as cows suffering from clinical milk fever. Herds were classified into negative (0 to 2/12), borderline (3 to 5/12), and positive ($\geq$ 6/12) according to the number of animals with hypocalcemia. Strategies to control hypocalcemia were documented. Prevalence of clinical milk fever was 1.4 %, 5.7 % and 16.1 % for second, third, and $\geq$ fourth parity cows, respectively. None of the cows in first lactation were suffering from clinical milk fever. Based on the threshold of 2.0 mmol/L 5.7 %, 29.0 %, 49.4 % and 60.4 % of cows in first, second, third, and $\geq$ fourth lactation were suffering from subclinical hypocalcemia, respectively. Fourteen, 51, and 50 herds were classified as negative, borderline, and positive, respectively. There was a positive association between serum calcium and serum phosphorus concentration. Serum calcium and magnesium concentration was negatively associated. Only 50 of 115 farms had a control strategy implemented to avoid hypocalcemia. Most common was the use of oral calcium products (40/115 herds), followed by feeding of anionic salts in the close-up diet (10/115 herds).

These results indicate that the prevalence of clinical and subclinical hypocalcemia in German dairy herds was high and that an active control strategy was not implemented on all farms. The negative association between calcium and magnesium warrants further research regarding the physiological regulation of these two minerals around parturition.

3.2 Key words

subclinical hypocalcemia, milk fever, parturition, magnesium

3.3 Introduction

Periparturient hypocalcemia is a common metabolic disorder in dairy cows, which leads to an increased risk of detrimental health and production outcomes and in severe case can be life-threatening. Physiologically, serum calcium concentration in the adult cow is maintained

above 2.0 mmol/L (Martin-Tereso and Martens, 2014). Due to the start of colostrum production and consequently increasing calcium demand, the nadir of serum calcium concentration occurs 12 to 24 hours after parturition (Goff, 2008; Kimura et al., 2006).

Hypocalcemia is considered as a gateway disease and predisposes the cow to various metabolic and infectious disorders in early lactation (Goff, 2008) such as metritis (Martinez et al., 2012) and mastitis (Curtis et al., 1983). In a study by Martinez et al. (2012), numbers of neutrophils were reduced and their ability to undergo phagocytosis and oxidative burst was impaired in cows affected by hypocalcemia which might in part explain the increased risk for infectious diseases. On a cellular level, suppressed function of immune cells was mediated by reduced cytosolic calcium concentration (Martinez et al., 2014).

Cows with naturally occurring hypocalcemia at parturition had elevated concentrations of NEFA and BHBA as indicators of increased lipomobilization (Martinez et al., 2012). The same group of authors were able to repeat these finding in cows with induced hypocalcemia (Martinez et al., 2014). Induction of hypocalcemia with EDTA infusion caused reduced dry matter intake and decreased plasma concentrations of insulin. These negative effects are supported by other studies showing an increased risk for displaced abomasum (Chapinal et al., 2011; Seifi et al., 2011), increased weight loss in early lactation (Caixeta et al., 2015), and ultimately an increased culling risk (Seifi et al., 2011; Roberts et al., 2012) for cows with hypocalcemia. Furthermore, subclinical hypocalcemia affected reproductive performance such as estrous cyclicity (Ribeiro et al., 2013; Caixeta et al., 2017) and pregnancy rate to first AI (Chapinal et al., 2012).

In a retrospective study including 1,462 cows from 480 dairy farms in 21 States of the US prevalence of hypocalcemia was 25 % in first lactation cows and about 50 % in multiparous cows (Reinhardt et al., 2011). Clinical milk fever was prevalent in 1 %, 4 %, 6 % and 10 % of first, second, third, and ≥ fourth lactation cows, respectively. These results originate from the 2002 NAHMS Dairy study (USDA, 2002). This study has been used as a reference for the prevalence of hypocalcemia multiple times. But the study was not specifically designed to estimate cow- and herd-level prevalence of hypocalcemia and the currentness of the results are limited.

More recently, different strategies (e.g., oral calcium supplementation, anionic salts) to prevent hypocalcemia have evolved and were implemented in the dairy industry (Martin-Tereso and Martens, 2014). These approaches might affect the prevalence of hypocalcemia. To our knowledge, however, there is no information available reporting the actual prevalence of hypocalcemia and associated preventive strategies.

Therefore, the objective of this study was to estimate the prevalence of hypocalcemia on a cow-level and the implemented preventive strategies to control for hypocalcemia in commercial German dairy herds.

3.4   Material and methods

The experimental procedures reported herein were conducted with the approval of the Institutional Animal Care and Use Committee of the Freie Universität Berlin.

3.4.1   Study population

A cross-sectional study was conducted based on a convenience sample of 115 dairy herds from eight federal states of Germany between February 2015 and August 2016. Inclusion criteria for herds were 1) participation in a federal DHIA testing system, 2) freestall housing with at least 100 milking cows, 3) feeding of a TMR-based diet, and 4) a computerized herd management software. Average herd size was 513 and ranged from 112 to 2,607 lactating cows. The average milk production (305 d ECM, 4.0 % fat, 3.4 % protein) was 9,231 kg (range 6,257 kg – 10,880 kg). Holstein Friesian cows were the dominant breed on 112 farms. Two farms kept Simmental cattle and one farm Jersey as the dominant breed.

A sample size calculation was conducted according to Dohoo et al. (2009). We assumed that the prevalence of milk fever tends to be highly clustered within herds because of the impact of herd management (e.g., breed, dry cow nutrition) on the risk of hypocalcemia. Therefore, we selected an intra-cluster correlation coefficient of 0.3. A sample size of 1,388 animals with 12 animals per herd was deemed adequate to estimate the true prevalence of subclinical hypocalcemia on a cow-level with 95 % confidence and 10 % precision.

If a farm provided less than twelve blood samples, the farm was excluded from statistical analysis. If a farm supplied more than 12 blood samples, 12 cows were selected, using a random function in Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany). A random list was generated separately for each of the farms.

3.4.2   Experimental procedures

Veterinary practitioners had been invited to participate in the study by an information leaflet sent out by regular mail. Participating practices were informed about the nature and

duration of the study and received a package containing serum blood collection systems (S-Monovette 9ml Z, Sarstedt AG & Co, Nürnbrecht, Germany), cryo-vials (Cryvial, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to store serum at -20 °C until analysis, and a written standard operating procedure (**SOP**). This SOP described which information to record for each cow enrolled and how to examine the cow before blood collection. A case report form for each cow was provided to document time of sampling, ear tag number, time of calving, calving ease (i.e., unassisted calving or assisted calving with at least one person), clinical symptoms of milk fever (i.e., recumbency), and parity. Administration of calcium products, time relative to calving and route of administration (i.e., subcutaneous, intravenous, oral) of these products was also documented. Sampling 12 cows per herd, veterinarians were asked to include four primiparous cows into the cohort. The farm personnel was asked, if other preventive strategies, such as feeding of anionic salts in the close-up group or injection of vitamin D before calving were implemented.

Animals were enrolled by convenience when a veterinarian visited the farm on a given day and an animal met the inclusion criteria of being within 48 h after parturition.

3.4.3   *Definition of hypocalcemia on cow-level and herd-level*

Normocalcemia was defined as serum calcium concentration greater or equal to 2.0 mmol/L (Reinhardt et at., 2011). Cows not affected clinically but with a serum calcium concentration below 2.0 mmol/L were categorized as subclinical hypocalcemic animals. Recumbent cows with a serum calcium concentration below 2.0 mmol/L were defined as cows suffering from clinical milk fever. Although often used in current literature (Reinhardt et al., 2011; Wilhelm et al., 2017), it was recently shown that 2.0 mmol/L is the most conservative approach as higher thresholds (i.e., 2.1 mmol/L and 2.2 mmol/L) were also associated with negative health or production outcomes (Chapinal et al., 2011; Seifi et al., 2011; Chapinal et al., 2012; Martinez et al. 2012; Roberts et al. 2012). Therefore, analyses were conducted considering three thresholds (i.e., 2.0 mmol/L, 2.1 mmol/L, 2.2 mmol/L).

Based on the results of the sampled cohort per farm, herds were categorized into negative (≤ 2 animals per herd with serum calcium concentration below the threshold), borderline (3 to 5 animals per herd with serum calcium concentration below the threshold) or positive (≥ 6 animals per herd with serum calcium concentration below the threshold) according to Cook et al. (2006). Using a confidence level of 75 % and an alarm level of 30 % the sampling of 12 animals per herd is adequate to classify herds into three categories (Figure 1).

3.4.4 *Blood sampling and laboratory analyses*

Blood samples were taken from the coccygeal vessels using a serum blood collection system. Samples were kept at room temperature and allowed to clot. Within 5 h of blood collection, samples were centrifuged to harvest serum, which was frozen at -20 °C. Analysis of blood samples was carried out by a commercial laboratory (Synlab Services GmbH, Augsburg, Germany). Total serum calcium, magnesium and phosphorus concentration was analyzed using photometry (AU680, Beckman Coulter, Krefeld, Germany). The interassay coefficient of variation was 1.03 % (Ca 2.37 mmol/L; n = 16), 1.06 % (Mg = 0.99 mmol/L; n = 16), and 2.43 % (P = 0.83 mmol/L; n = 16) for calcium, magnesium and phosphorus, respectively. The intraassay coefficient of variation was 1.19 % (Ca 2.40 mmol/L; n = 10), 0.88 % (Mg = 0.99 mmol/L; n = 10), and 1.03 % (P = 0.85 mmol/L; n = 10) for calcium, magnesium and phosphorus, respectively.

3.4.5 *Statistical analyses*

Individual cow data were transferred to Microsoft Excel (Office 2013, Microsoft Deutschland Ltd., Munich, Germany). Statistical analyses were performed using SPSS for Windows (version 22.0, SPSS Inc., IBM, Ehningen, Germany). The association of lactation number and the type of hypocalcemia or the type of individual preventive strategy was analyzed using cross tabulations and χ^2 tests.

For evaluation of the association between serum calcium concentration and time of calving, ease of calving, and time interval from calving to sampling, we used the GENLINMIXED procedure of SPSS. Cow was the experimental unit and herd was considered as a random effect. According to the model-building strategies described by Dohoo et al. (2009) each parameter considered for the mixed model should be separately analyzed in a univariate model, including the parameter as a fixed factor (i.e., categorical parameter) or covariate (i.e., continuous parameter). Only parameters resulting in univariate models with $P \leq 0.2$ should be included in the final mixed model. The initial model contained the following explanatory variables as fixed effects: parity (1, 2, 3, greater or equal to 4), breed (Holstein, Jersey, or Simmental), time of calving (day time from 6:00 am to 5:59 pm vs. night time 6:00 pm to 5:59 am), calving ease (unassisted calving vs. assisted calving), and time interval from calving to sampling (continuous; 0 to 48 h).

The concentration of calcium was related to the concentration of phosphorus or magnesium using a linear regression model and the LINEAR REGRESSION procedure from SPSS: $y_i = a + bX_i$, where y_i is the dependent variable (magnesium or phosphorous

concentration), Xi is the independent variable (calcium concentration), b is the slope of the regression line, and a is the intercept. R^2 describes the coefficient of determination, which is the relative proportion of variance in yi that can be explained by Xi.

3.5   Results

Overall, blood samples were drawn from 1,709 animals at 0 to 48 h after calving from 125 farms. Ten farms with 54 animals were excluded from analysis because they provided less than 12 samples per farm. Another 275 animals were randomly excluded because 60 farms provided more than 12 samples per farm.

Data of 1,380 animals were available for final analyses. Of those, 228 (16.5 %), 355 (25.7 %), 332 (24.1 %), and 465 (33.7 %) were in first, second, third, and ≥ fourth lactation, respectively.

Based on a calcium threshold of 2.0 mmol/L and clinical signs, the prevalence of subclinical hypocalcemia and clinical milk fever was 40.7 % (561/1,380) and 7.2 % (99/1,380), respectively. Considering higher thresholds of 2.1 mmol/L and 2.2 mmol/L, prevalence of subclinical hypocalcemia increased to 53.0 % (732/1380) and 67.5 % (931/1380), respectively (Table 1). The prevalence of hypocalcemia increased with parity. None of the cows in first lactation was suffering from clinical milk fever. Prevalence of clinical milk fever was 1.4 % (5/355), 5.7 % (19/332) and 16.1 % (75/465) for second, third, and ≥ fourth parity cows, respectively (Table 1).

There was a significant effect of parity ($P < 0.001$) on serum calcium concentration. Cows being in first, second, third or ≥ fourth lactation had a serum calcium concentration of 2.213 mmol/L (95 % CI: 2.054 – 2.372), 2.102 mmol/L (95 % CI: 1.942 – 2.262), 1.997 mmol/L (95 % CI: 1.837 – 2.158), and 1.891 mmol/L (95 % CI: 1.735 – 2.046), respectively. Serum calcium concentration of cows that calved at night was 0.087 mmol/L higher compared with cows that calved during the day (95 % CI: 0.044 – 0.130; $P = 0.001$). There was no significant effect of breed ($P = 0.811$), time from calving to sampling ($P = 0.288$) and calving ease ($P = 0.902$) on serum calcium concentration.

A negative association of serum calcium and serum magnesium concentration was observed (y = -0.208x + 1.454; $R^2 = 0.151$; $P < 0.001$; Figure 2). In contrast, there was a positive association of serum calcium and serum phosphorus concentration (y = 0.900x – 0.229; $R^2 = 0.335$; $P < 0.001$; Figure 3).

Prevention of hypocalcemia on a cow-level was more prevalent in multiparous cows compared to primiparous cows. Oral calcium supplementation was implemented in 13.8 %, 24.1 % and 26.0 % in second, third, and ≥ fourth parity cows (Table 2) and most prevalent for prevention of hypocalcemia at the cow-level (n = 255; Table 2). Subcutaneous calcium injection or prepartum vitamin D application played a minor role and was on the herd-level always combined with another preventive strategy (Table 2). In 34.8 % (40/115), 6.1 % (7/115), and 2.6 % (3/115) of the herds, oral calcium supplementation, anionic salts, or a combination of both was used to control hypocalcemia on a herd-level, respectively. Most of the herds (65/115) did not implement a control strategy for hypocalcemia.

Only 12.2 % of the 115 herds enrolled were classified as negative based on the alarm levels set by Cook et al. (2006).
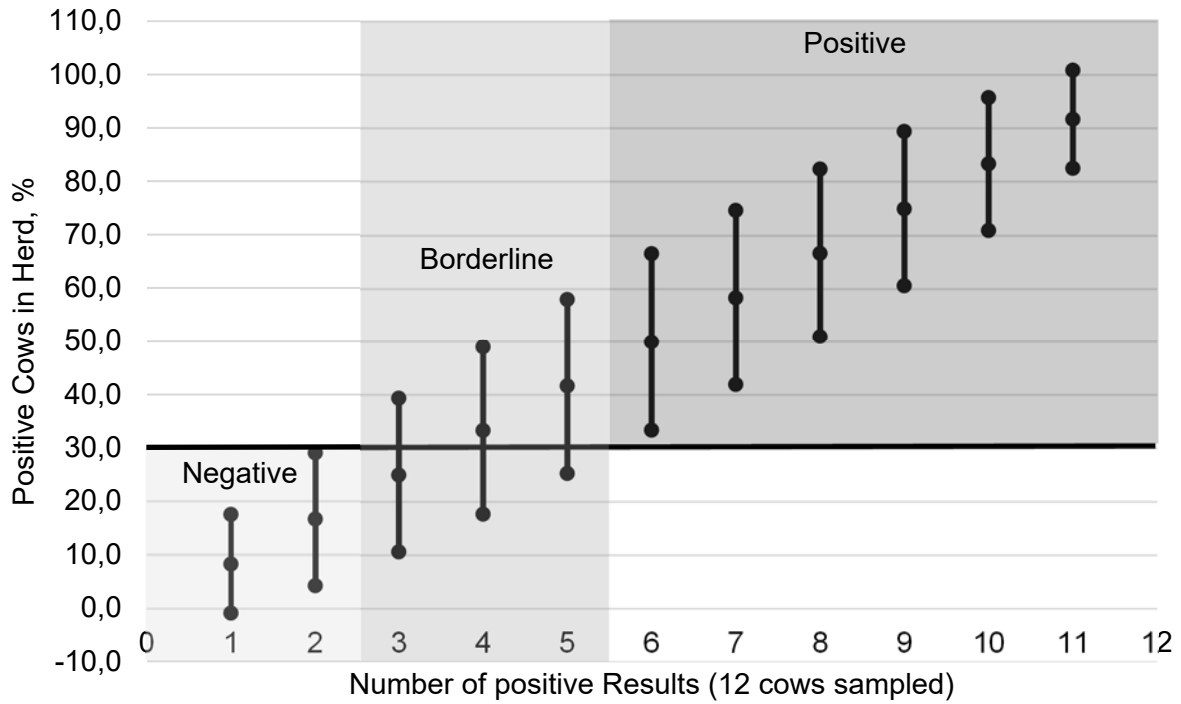
Figure 1. Classification of blood calcium concentrations using 75 % confidence intervals and an alarm level of 30 % for test results from 12 cows sampled from a group of 100 cows. This calculation illustrates the association between positive blood samples in the cohort and prevalence of hypocalcemia in the tested herd.
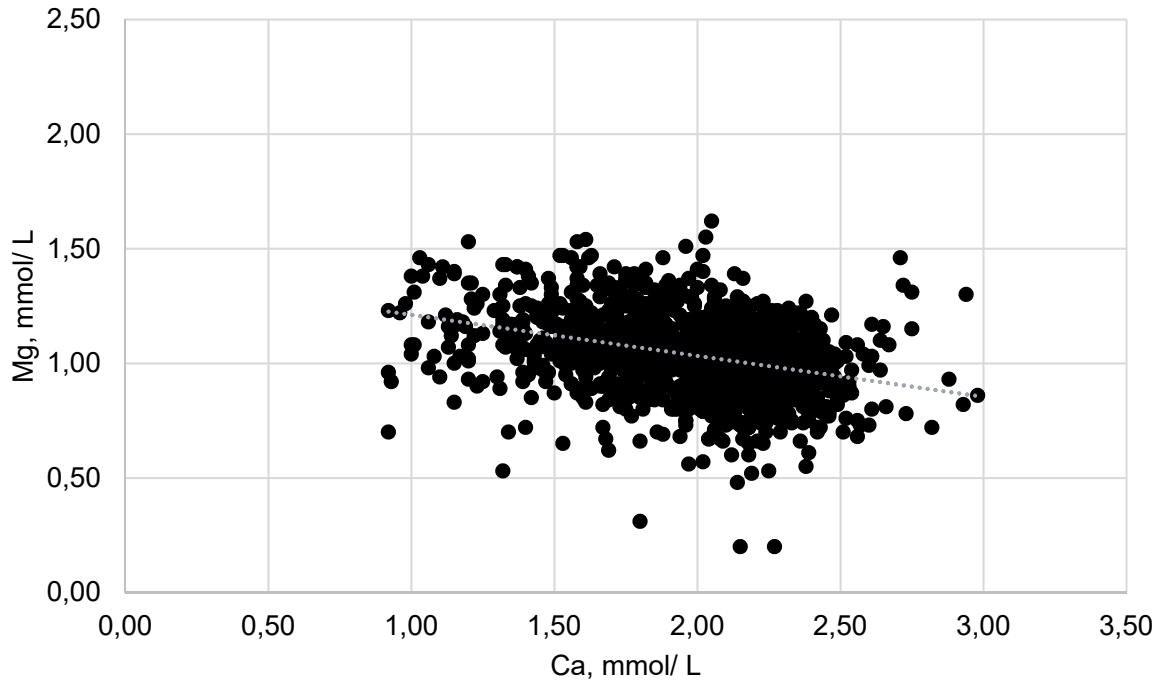
Figure 2. Association between serum calcium and serum magnesium concentration for all cows (n = 1,380, y = −0.208x + 1.454; R^2 = 0.151; *P* < 0.001).
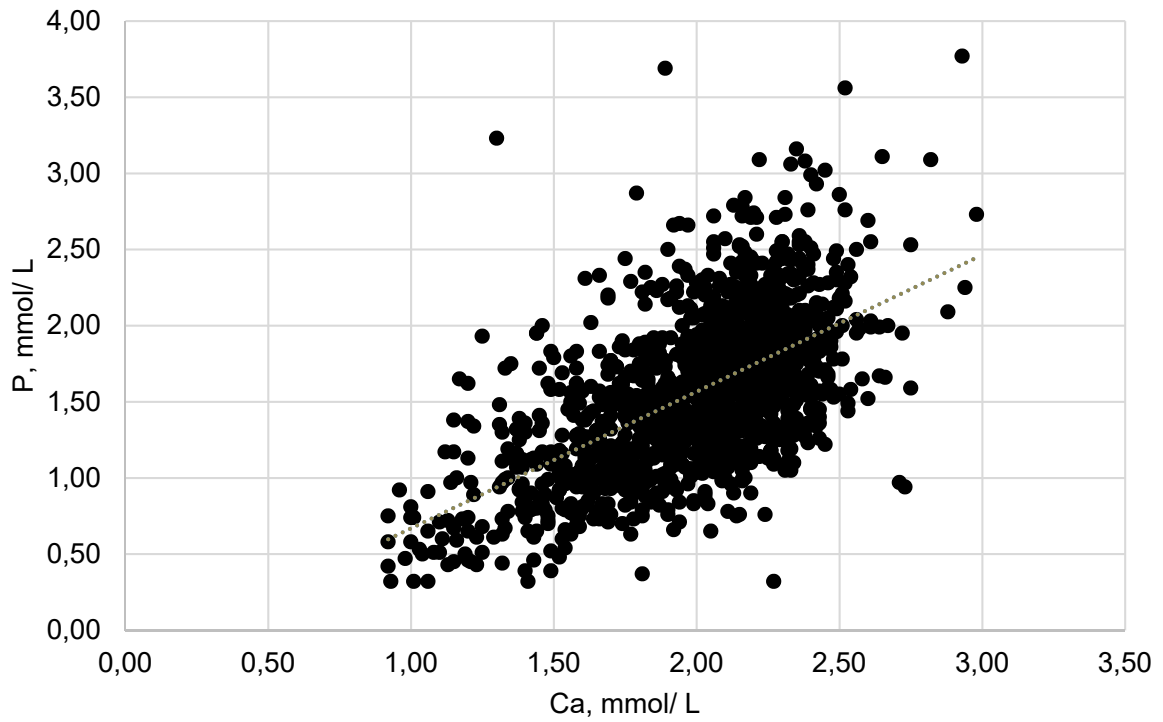
Figure 3. Association between serum calcium and serum phosphorus concentration for all cows (n = 1,380, y = 0.900x − 0.229; R^2 = 0.335; *P* < 0.001).

Table 1. Prevalence of subclinical hypocalcemia and milk fever 0 to 48 h after parturition in dairy cows stratified by parity considering three thresholds for blood calcium.

| Type of hypocalcemia	Lactation 1	Lactation 2	Lactation 3	Lactation ≥ 4
Subclinical				
Threshold 2.0 mmol/L	13/228[a]	103/355[b]	164/332[c]	281/465[d]
	5.7 %	29.0 %	49.4 %	60.4 %
Threshold 2.1 mmol/L	32/228[a]	158/355[b]	211/332[c]	331/465[d]
	14.0 %	44.5 %	63.6 %	71.2 %
Threshold 2.2 mmol/L	83/228[a]	222/355[b]	256/332[c]	370/465[b,c]
	36.4 %	62.5 %	77.1 %	79.6 %
Clinical	0/228[a]	5/355[a]	19/332[b]	75/465[c]
	0.0 %	1.4 %	5.7 %	16.1 %

Different superscripts within rows differ $P < 0.05$.

Table 2. Type of prophylaxis to prevent hypocalcemia in 1,380 cows of 115 dairy herds considering parity.

Type of prophylaxis	Lactation 1	Lactation 2	Lactation 3	Lactation ≥ 4
Oral Calcium	5/228[a]	49/355[b]	80/332[c]	121/465[c]
	2.2 %	13.8 %	24.1 %	26.0 %
Subcutaneous Calcium	0/228[a]	7/355[a,b]	14/332[b]	23/465[b]
	0.0 %	2.0 %	4.2 %	4.9 %
Vitamin D prepartum	0/228[a,b]	0/355[b]	7/332[a,c]	15/465[c]
	0.0 %	0.0 %	2.1 %	3.2 %
Overall	5/228	56/355	101/332	159/465
	2.2 %	15.8 %	30.4 %	34.2 %

Different superscripts within rows differ $P < 0.05$.

3.6   Discussion

The results from the present study indicate that the prevalence of hypocalcemia within 48 h after parturition in TMR fed, freestall dairy herds in Germany was high, particularly in multiparous cows. In contrast, preventive strategies were not consistently implemented on the farms.

Cows with a serum calcium concentration below 2.0 mmol/L were considered as hypocalcemic. Although this is a conservative threshold, it is well accepted in research and applied in the field (Degaris et al., 2009; Reinhardt et al., 2011; Wilhelm et al., 2017). Recent studies suggested higher thresholds. It was shown that hypocalcemia using 2.1 mmol/L, 2.2 mmol/L, and 2.3 mmol/L thresholds was associated with a negative health outcome such as displaced abomasum and metritis (Chapinal et al., 2011; Chapinal et al., 2012; Martinez et al., 2012) or an increased culling risk (Seifi et al., 2011; Roberts et al., 2012). These studies, however, considered a longer risk period postpartum, i.e., of 3 to 7 DIM. While these epidemiological studies showed an association and also some evidence for a causal relationship between hypocalcemia and an increased risk for infectious diseases (Martinez et al., 2014) one has to be careful when evaluating longer risk periods. It is also plausible that reduced feed intake before clinical signs of disease can affect serum calcium levels as most recently shown by Pinedo et al. (2017) for cows suffering from puerperal metritis. At calving, there was no difference in serum calcium concentration between healthy and metritic cows. On the day of diagnosis (on average 6.1 DIM), however, serum calcium concentration was lower in cows with puerperal metritis (1.57 mmol/L) compared with healthy cows (2.10 mmol/L). In order to reflect higher thresholds, we calculated the prevalence accordingly. Table 1 shows that a mild increase of the threshold leads to a dramatic increase of animals considered as hypocalcemic. Further evaluations are necessary to define the most appropriate threshold of hypocalcemia within this time period.

In our study, 47.6 % of multiparous cows suffered from subclinical hypocalcemia within 48 h after parturition. This finding is in agreement with others (Reinhardt et al., 2011; Gild et al., 2015; Miltenburg et al., 2015). The inability of the cow to maintain normal serum calcium concentration is caused by a maladaptation of the mineral metabolism in response to an increased demand for calcium. Dairy cows need around 20 grams of calcium per day at the end of the dry period. With colostrum production, the demand increases to 30 to 70 grams per day depending on milk yield. The mechanisms to decrease urinary calcium excretion, to increase absorption of calcium from the gut, and to upregulate calcium release from bone tissue, however, take about 48 h which may lead to insufficient calcium supply in this period (Martin-Tereso and Martens, 2014).

Surprisingly, serum calcium concentration of cows that calved at night was higher than serum calcium concentration of cows that calved during the day. The effect, however, was biologically small and without an apparent explanation.

In contrast to multiparous cows, hypocalcemia was rarely found in primiparous cows (5.7 %) which is in agreement with a previously described prevalence of 2 % for primiparous cows from seven herds in Canada using the same threshold (Miltenburg et al., 2015). Overall, there are, however, conflicting reports on the prevalence of hypocalcemia in primiparous cows. Reinhardt et al. (2011) observed a prevalence of 25 % from 480 herds in the US. The reason for this difference remains speculative. The latter report utilized samples drawn for the 2002 NAHMS study. The understanding of the transition cow biology improved in the last 15 years and led to the implementation of preventive strategies to control milk fever. As stated in the NAHMS 2002 report (USDA, 2002), 14.3 % of heifers and 19.1 % of cows were fed anionic salts in their close-up diets. In the more recent NAHMS report, 20.7 % and 27.6 % of heifers and cows were fed anionic salts, respectively (USDA, 2014). This might be an indication of an increased awareness for prevention of milk fever and subclinical hypocalcemia. Furthermore, management practices and production conditions for heifers might have changed in the meantime (e.g., close-up feeding, milk yield, feed intake).

A plausible explanation for the higher prevalence of subclinical hypocalcemia in multiparous cows is that these animals have a higher calcium output due to an increased amount of colostrum compared to primiparous cows (Klingbeil, 2015). The calcium output, however, does not explain the increase in prevalence of hypocalcemia within multiparous cows with increasing age as their colostrum yield was not different. The parity associated increase might be related to a reduced bone remodeling in multiparous cows due to a reduction of the number of active osteoclasts and osteoblasts. These cells must be recruited from progenitor cells in response to parathyroid hormone (**PTH**) secretion leading to a delay in calcium mobilization (Goff, 2014).

In a recent study, 51 %, 54 %, and 42 % of fourth, fifth, and sixth parity cows suffered from subclinical hypocalcemia, respectively (Reinhardt et al., 2011). These results are almost identical with our observations of 52.1 %, 51.1 %, and 41.7 % for fourth, fifth, and sixth parity cows, respectively. Prevalence of clinical milk fever in our data set (13.4 %, 15.0 %, and 21.7 % for fourth, fifth, and ≥ sixth parity), however, was higher than described for the US (10 %, 8 %, and 13 % for fourth, fifth, and sixth parity (Reinhardt et al., 2011)). We assume that preventive strategies are more common in the US. In the present study, calcium was supplemented orally (40 herds) or subcutaneously (13 herds) in 46.1 % of the collaborating herds. Only 8.7 % of the herds used anionic salts to prevent hypocalcemia. In the US, calcium supplementation also seems to be the most common strategy to prevent hypocalcemia. Based

on the recent NAHMS report, 68.9 % and 27.6 % of the participating herds used calcium products and anionic salts, respectively (USDA, 2014). A trend was observed for large dairy farms (≥ 500 milking cows) to use calcium supplementation and anionic salts more often than smaller operations.

German farmers favored oral calcium supplementation as preventive strategy (Table 2). There is sound evidence about the efficacy of oral calcium supplementation in order to prevent hypocalcemia (Sampson et al., 2009; Oetzel and Miller, 2012; Blanc et al., 2014; Martinez et al., 2016). Furthermore, economic advantages have been demonstrated (McArt and Oetzel, 2015). Based on a simulation with 1,000 calvings per year, a farm can expect an average net gain ranging from $3,000 to $8,000 after postpartum supplementation of oral calcium to multiparous animals. In the present study, cows in their third or greater lactation were predominantly supplemented with oral calcium products. It is noteworthy, however, that dairy farmers did not consider higher risk for hypocalcemia in older animals as the percentage of supplemented cows was almost the same for cows in lactation 3 (30 %) or higher (34 %).

Calving ease had no significant effect on serum calcium concentration. Since there is evidence, that primiparous cows have higher serum calcium concentrations, this finding might be confounded as presence of dystocia is more prevalent in primiparous cows (Mee, 2004; USDA, 2010).

Comparing different breeds, serum calcium concentration did not differ among Holstein, Simmental and Jersey cows. These results must be interpreted with caution, as only one and two farms kept Jersey and Simmental cows as their dominant breed, respectively.

Serum calcium and magnesium concentration were negatively associated. Cows suffering from hypocalcemia had higher serum magnesium concentration (Figure 2). This seems to be contradictory as magnesium is considered as an important prerequisite for proper PTH-receptor function (Goff, 2014). Renal excretion of magnesium depends on oral magnesium intake and the concentration of PTH (Martin-Tereso and Martens, 2014). In a period of low serum calcium concentration, PTH is secreted into the blood, leading to calcium retention in the kidneys and increased resorption of calcium in the gut via production of 1,25-dihydroxyvitamin D. Our findings are in agreement with a previous assumption, that PTH secretion raises the threshold for renal magnesium excretion resulting in a higher serum magnesium concentration (Goff, 2008).

In contrast, serum phosphorus concentration was low in cows suffering from hypocalcemia (Figure 3). Mechanisms of phosphorus regulation are not fully understood yet. Also, the role of hypophosphatemia in recumbent cows remains speculative. A 21 month long feeding trial with a diet restricted in phosphorus (2.4 g/kg) led to reduced dry matter intake,

milk yield, and body weight, but did not result in recumbency (Valk et al., 1999). Recently, it has been shown, however, that the presence of PTH in hypocalcemic cows increased phosphorus excretion through urine and saliva (Grünberg, 2014). A reduction of dry matter intake prepartum can also cause insufficient phosphorus uptake through the diet and contribute to phosphorus depletion. Similar to the decrease of calcium around parturition, the major reason for hypophosphatemia is the production of colostrum and milk, as approximately 1 g of phosphorus is excreted in every kilogram of milk (Goff, 1999; Grünberg, 2014).

3.7   Study limitations

The objective of this study was to characterize the cow-level prevalence of clinical and subclinical hypocalcemia in German dairy herds. To achieve this objective, 115 farms of 8 federal states from Germany were enrolled through veterinary practitioners that had been asked to collaborate in this multi-center study. As many other studies determining prevalences for hyperketonemia (e.g., Ospina et al., 2010; Chapinal et al., 2012; Suthar et al., 2013) or hypocalcemia (Gild et al., 2015; Miltenburg et al., 2015) the dairy farms enrolled constitute a convenience sample. It is difficult to conclude if the participating dairies are representative for all dairies of the given region. In fact, the selection of herds might have been biased by an underlying interest to participate stimulated by a previous history with the condition at hand. The objective was not to compare within herd-level prevalences of hypocalcemia, as the confidence interval for the prevalence estimate is wide using only 12 samples. Rather, we wanted to categorize herds based on the proportion of positive samples (i.e., blood calcium below threshold) into negative (0 to 2/12), borderline (3 to 5/12), or positive (≥ 6/12). Such classification is appropriate using 12 cows per herd based on the assumptions provided by Oetzel (2004) using a 75 % confidence interval and an alarm level of 30 % (Figure 1). For practitioners or consultants, this might be an adequate approach to screen a herd for the presence of subclinical hypocalcemia. As obvious from our results many herds were classified as borderline (44.3 %). In such case, we would advise to draw more samples in order to classify the herd more appropriately. Although the probability to misclassify a herd decreases with increasing sample size, it is the nature of the disease with a very short risk period (0 to 48 h) that limits this approach. In a 1,000 cow dairy, there are only 5.5 animals at risk to being sampled for evaluation of hypocalcemia on any given day.

Our study design is not appropriate to give a well-founded statement on blood calcium dynamics postpartum, due to the sampling time of 0 to 48 hours postpartum and frequency of sampling (one sample per cow). Recent studies with a repeated measurement in individual

cows (Blanc et al., 2014; Caixeta et al., 2017) are more suitable to illustrate the effect of time of sampling on serum calcium concentration.

Based on our inclusion criteria, small dairy farms (< 100 cows per farm) were not investigated. Therefore, this study might underestimate the true prevalence of hypocalcemia as the implementation of preventive strategies was more common in large dairy operations in the US (USDA, 2014).

In order to represent common herd demographics, veterinarians were asked to include approximately one third of primiparous cows in the samples per farm. In our study, 16.5 % of the sampled animals were primiparous cows. This is not representative for the participating German dairy herds as the average percentage of primiparous cows per herd was 32.2% and the overall percentage of primiparous cows in Germany was 35.5 % (Arbeitsverband Deutscher Rinderzüchter, 2016). A possible explanation for this disagreement might be, that participating veterinarians were aware that hypocalcemia is more prevalent in multiparous cows and therefore preferred to sample these cows. As indicated by our results, primiparous cows did not suffer from clinical milk fever at all and rarely from subclinical hypocalcemia. In order to estimate the level of hypocalcemia on a farm, we would recommend to focus on multiparous cows to reflect their higher risk to suffer from hypocalcemia.

3.8   Conclusions

Based on a convenience sample using 115 TMR fed, freestall herds from Germany with 1,380 cows sampled within 48 h after parturition, the prevalence of hypocalcemia was estimated. Overall, the present study indicates that periparturient hypocalcemia is a prevalent metabolic disorder on dairy farms as 47.6 % and 8.6 % of multiparous cows suffered from subclinical hypocalcemia and clinical milk fever, respectively. For primiparous cows, the prevalence of subclinical hypocalcemia was 5.7 %. An active control strategy was implemented only on 43.5% of the farms. Oral calcium supplementation at parturition was the most predominant preventive strategy, particularly in multiparous cows (21.7 %). The negative association between calcium and magnesium was an interesting finding that warrants further research into the physiologic regulation of these two minerals at parturition. The results of this study are based on a large number of herds with different managerial conditions and seem to be applicable to high producing dairy herds.

3.9 Acknowledgements

3.10 References

Arbeitsverband Deutscher Rinderzüchter. 2016. Rinderproduktion in Deutschland 2015. Arbeitsgemeinschaft Deutscher Rinderzüchter e.V. (ADR), Bonn, Germany.

Blanc, C. D., M. Van der List, S. S. Aly, H. A. Rossow, and N. Silva-del-Rio. 2014. Blood calcium dynamics after prophylactic treatment of subclinical hypocalcemia with oral or intravenous calcium. J. Dairy Sci. 97:6901-6906.

Caixeta, L. S., P. A. Ospina, M. B. Capel, and D. V. Nydam. 2015. The association of subclinical hypocalcemia, negative energy balance and disease with bodyweight change during the first 30 days post-partum in dairy cows milked with automatic milking systems. Vet. J. 204:150-156.

Caixeta, L. S., P. A. Ospina, M. B. Capel, and D. V. Nydam. 2017. Association between subclinical hypocalcemia in the first 3 days of lactation and reproductive performanceof dairy cows. Theriogenology. doi: 10.1016/j.theriogenology.2017.01.039.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. J. Dairy Sci. 94:4897-4903.

Chapinal, N., M. E. Carson, S. J. LeBlanc, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012. The association of serum metabolites in the transition period with milk production and early-lactation reproductive performance. J. Dairy Sci. 95:1301-1309.

Cook, N., G. Oetzel, and K. Nordlund. 2006. Modern techniques for monitoring high-producing cows 2. Practical applications. In Pract. 28:598-603.

Curtis, C. R., H. N. Erb, C. J. Sniffen, R. D. Smith, P. A. Powers, M. C. Smith, M. E. White, R. B. Hillman, and E. J. Pearson. 1983. Association of parturient hypocalcemia with eight periparturient disorders in Holstein cows. J. Am. Vet. Med. Assoc. 183:559-561.

DeGaris, P. J. and I. J. Lean. 2008. Milk fever in dairy cows: a review of pathophysiology and control principles. Vet. J. 176:58-69.

Dohoo, I. R., S. W. Martin, and H. Stryhn. 2009. Veterinary Epidemiologic Research. 2nd ed. University of Prince Edward Island, Charlottetown, PEI, Canada.

Gild, C., N. Alpert, and M. van Straten. 2015. The Influence of SCH on Milk Production and Reproduction in israeli dairy herds. Isr. J. Vet. Med. 70:16-21.

Goff, J. P. 1999. Treatment of calcium, phosphorus, and magnesium balance disorders. Vet. Clin. North Am. Food Anim. Pract. 15:619-639.

Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Vet. J. 176:50-57.

Goff, J. P. 2014. Calcium and magnesium disorders. Vet. Clin. North Am. Food Anim. Pract. 30:359-381.

Grünberg, W. 2014. Treatment of phosphorus balance disorders. Vet. Clin. North Am. Food Anim. Pract. 30:383-408.

Kimura, K., T. A. Reinhardt, and J. P. Goff. 2006. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. J. Dairy Sci. 89:2588-2595.

Klingbeil, M. 2015. Investigation of influence factors on yield, quality and calcium content of first colostrum in Holstein. Doctoral thesis. Ruminant and Swine Clinic, Faculty of Veterinary Medicine, Freie Universität Berlin, Germany

Martin-Tereso, J. and H. Martens. 2014. Calcium and magnesium physiology and nutrition in relation to the prevention of milk fever and tetany (dietary management of macrominerals in preventing disease). Vet. Clin. North Am. Food Anim. Pract. 30:643-670.

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvao, and J. E. Santos. 2012. Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. J. Dairy Sci. 95:7158-7172.

Martinez, N., L. D. Sinedino, R. S. Bisinotto, E. S. Ribeiro, G. C. Gomes, F. S. Lima, L. F. Greco, C. A. Risco, K. N. Galvao, D. Taylor-Rodriguez, J. P. Driver, W. W. Thatcher, and J. E. Santos. 2014. Effect of induced subclinical hypocalcemia on physiological responses and neutrophil function in dairy cows. J. Dairy Sci. 97:874-887.

Martinez, N., L. D. Sinedino, R. S. Bisinotto, R. Daetz, C. A. Risco, K. N. Galvao, W. W. Thatcher, and J. E. Santos. 2016. Effects of oral calcium supplementation on productive and reproductive performance in Holstein cows. J. Dairy Sci. 99:8417-8430.

McArt, J. A. and G. R. Oetzel. 2015. A stochastic estimate of the economic impact of oral calcium supplementation in postparturient dairy cows. J. Dairy Sci. 98:7408-7418.

Mee, J. F. 2004. Managing the dairy cow at calving time. Vet. Clin. North Am. Food Anim. Pract. 20:521–546.

Miltenburg, C. L. 2015. Management of peripartum dairy cows for metabolic health and immune function. Doctoral Thesis. Department of Population Medicine, University of Guelph, Ontario, Canada.

Oetzel, G. R. 2004. Monitoring and testing dairy herds for metabolic disease. Vet. Clin. North Am. Food Anim. Pract. 20:651-674.

Oetzel, G. R. and B. E. Miller. 2012. Effect of oral calcium bolus supplementation on early-lactation health and milk yield in commercial dairy herds. J. Dairy Sci. 95:7051-7065.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010. Associations of elevated nonesterified fatty acids and beta-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. J. Dairy Sci. 93:1596-1603.

Pinedo, P., J. Velez, G. Solano, N. Rodriguez, J. Naves, G. M. Schuenemann and, C. Riscoll. 2017. Effect of oral calcium administration on the cure and reproductive performance of Holstein cows diagnosed with puerperal metritis. J. Dairy Sci. doi: 10.3168/jds.2016-11577.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Vet. J. 188:122-124.

Ribeiro, E. S., F. S. Lima, L. F. Greco, R. S. Bisinotto, A. P. Monteiro, M. Favoreto, H. Ayres, R. S. Marsola, N. Martinez, W. W. Thatcher, and J. E. Santos. 2013. Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. J. Dairy Sci. 96:5682-5697.

Roberts, T., N. Chapinal, S. J. Leblanc, D. F. Kelton, J. Dubuc, and T. F. Duffield. 2012. Metabolic parameters in transition cows as indicators for early-lactation culling risk. J. Dairy Sci. 95:3057-3063.

Sampson, J. D., J. N. Spain, C. Jones, and Carstensen, L. 2009. Effects of calcium chloride and calcium sulfate in an oral bolus given as a supplement to postpartum dairy cows. Vet. Ther. 10:131-139.

Seifi, H. A., S. J. Leblanc, K. E. Leslie, and T. F. Duffield. 2011. Metabolic predictors of post-partum disease and culling risk in dairy cattle. Vet. J. 188:216-220.

Suthar, V. S., J. Canelas-Raposo, A. Deniz, and W. Heuwieser. 2013. Prevalence of subclinical ketosis and relationships with postpartum diseases in European dairy cows. J. Dairy Sci. 96:2925-2938.

USDA. 2002. Part I: Reference of Dairy Cattle Health and Health Management Practices in the United States. APHIS, Fort Collins, CO.

USDA. 2010. Dairy 2007, Heifer Calf Health and Management Practices on U.S. Dairy Operations. APHIS, Fort Collins, CO.

USDA. 2014. Dairy Cattle Management Practices in the United States. APHIS, Fort Collins, CO.

Valk, H. and L. B. Sebek. 1999. Influence of long-term feeding of limited amounts of phosphorus on dry matter intake, milk production, and body weight of dairy cows. J. Dairy Sci. 82:2157-2163.

Wilhelm, A. L., M. G. Maquivar, S. Bas, T. A. Brick, W. P. Weiss, H. Bothe, J. S. Velez, and G. M. Schuenemann. 2017. Effect of serum calcium status at calving on survival, health, and performance of postpartum Holstein cows and calves under certified organic management. J. Dairy Sci. doi: 10.3168/jds.2016-11743.

4   ADDITIONAL UNPUBLISHED WORK

Impact of periparturient hypocalcemia on milk yield, reproductive performance and culling in dairy cows

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4.1 Abstract

Periparturient hypocalcemia is frequently observed and considered as a gateway disease that predisposes the cow to various other diseases or health issues. The objective of this study was to evaluate the impact of hypocalcemia on milk yield, reproductive performance, and culling across a large number of different managerial systems. A cross-sectional study was conducted based on a convenience sample of 125 dairy herds from 8 federal states of Germany between February 2015 and August 2016. Blood samples were drawn from 1,709 animals within 48 hours after parturition and analyzed for serum calcium concentration. After discarding cows (n = 283) with missing data, a total of 1,426 cows were considered for final analyses. For each herd, a record of the herd management software was requested 150 days after the last cow was sampled. Serum calcium concentration of each cow was related to early lactation milk yield (test day 1 to 3), reproductive performance (DIM at first artificial insemination (**AI**), pregnancy per AI at first AI, time to pregnancy within 150 DIM) and culling (until 60 DIM) data. Generalized linear mixed models were used to analyze continuous or categorical data. Shared frailty models were used for time to event data. Five different thresholds were used to define hypocalcemia. Thresholds ranged from 1.8 to 2.2 mmol/L using 0.1 mmol/L increments. Milk fever was defined as serum calcium concentration below 2.0 mmol/L in combination with clinical signs (e.g. recumbency). The effect of hypocalcemia on milk yield was conditional on parity. Primiparous cows suffering from hypocalcemia (threshold: 2.0 mmol/L) tended to produce 2.07 kg/d (P = 0.063) less milk compared with normocalcemic primiparous cows in early lactation. There was a tendency for hypocalcemic multiparous cows (threshold 2.1 mmol/L) to produce 0.51 kg/d (P = 0.086) more milk compared with normocalcemic cows. Multiparous cows suffering from clinical milk fever produced 1.90 kg/d (P = 0.002) less milk compared with normocalcemic cows in early lactation. Calcium status did not influence days to first insemination. Cows suffering from hypocalcemia (threshold 1.9 mmol/L) had decreased odds (OR 0.56; P = 0.001) of pregnancy at first artificial insemination. Calcium status had a significant impact on time to pregnancy (threshold 1.8 mmol/L). In comparison to normocalcemic animals, the hazard of becoming pregnant within 150 DIM was reduced when cows had a serum calcium concentration below 1.8 mmol/L (HR = 0.69; P = 0.001). Cows with hypocalcemia (threshold: 2.0 mmol/L) and cows suffering from milk fever had a 1.6 (P = 0.019) and 1.9 (P = 0.029) times greater hazard of being culled within the first 60 DIM compared with nornmocalcemic animals. Given the high prevalence of hypocalcemia and the associated negative downstream outcomes, this study re-emphasizes the need to establish control strategies for hypocalcemia.

4.2 Key words:

hypocalcemia, milk fever, reproduction, dairy cow

4.3 Introduction

Transition cows face the challenge of an increased requirement for minerals, especially calcium, to support lactogenesis in early lactation (Goff, 2014). In order to compensate this challenge, homeorhetic mechanisms take place to adapt for such increased demands (Martin-Tereso and Martens, 2014). Unsuccessful adaptation leads to hypocalcemia around parturition and has been associated with increased occurrence of diseases (Martinez et al., 2012), decreased milk production (Chapinal et al., 2012b), increased culling risk (Seifi et al., 2011; Roberts et al., 2012), and impaired reproductive performance (Martinez et al., 2012; Ribeiro et al., 2013; Caixeta et al., 2017). The major risk period for hypocalcemia is 0 to 24 hours after parturition (Kimura et al., 2006). Hypocalcemia can be either clinical (i.e., milk fever) or subclinical. With a better understanding of the hormonal regulation of calcium mobilisation in the periparturient cow (Goff, 2008) and appropriate nutritional management (e.g., DCAD) during the dry period and early lactation, incidence rates of clinical milk fever can be as low as 1 % (Oetzel and Miller, 2012). In a recent multi-site study comprising 115 herds in Germany, however, the incidence rate of milk fever averaged 7.2 % (Venjakob et al., 2017).

Subclinical hypocalcemia has been defined using thresholds ranging from 2.0 to 2.3 mmol/L (Seifi et al., 2011; Chapinal et al., 2012b; Roberts et al., 2012; Wilhelm et al., 2017). Previously, the threshold value to consider a cow as hypocalcemic was 2.0 mmol/L. This conservative threshold was well accepted in research and has often been applied in the field (DeGaris and Lean, 2008; Reinhardt et al., 2011; Wilhelm et al., 2017). The origin of this threshold, however, was somehow arbitrary and recent studies have reported higher thresholds. It was shown that hypocalcemia using the thresholds 2.1 mmol/L, 2.2 mmol/L, or 2.3 mmol/L was associated with a negative health outcome such as displaced abomasum and metritis (Chapinal et al., 2011; Chapinal et al., 2012b; Martinez et al., 2012) or an increased culling risk (Seifi et al., 2011; Roberts et al., 2012). These studies, however, considered a longer risk period postpartum, i.e., 3 to 7 DIM.

The impact of hypocalcemia on milk production has been controversially described. While one study demonstrated an association of hypocalcemia with decreased milk yield (Chapinal et al., 2012b), others did not show an effect (Martinez et al., 2012) and some studies even suggested that cows suffering from subclinical hypocalcemia produce more milk during early lactation (Jawor et al., 2012; Gild et al., 2015) compared with normocalcemic cows.

It has been shown consistently that the risk for culling during the first 60 days of lactation was greater when serum calcium concentration was ≤ 2.2 mmol/L in the first week postpartum (OR=1.5; 95 % CI = 1.2 to 1.9; Roberts et al., 2012). Other authors found a 2.4 and 5.3 times greater risk for culling, when serum calcium concentration was ≤ 2.2 mmol/L and ≤ 2.3 mmol/L in first and second week postpartum, respectively (Seifi et al., 2011).

Conclusively, there are some epidemiological multi-site studies indicating a negative impact of hypocalcemia in the first week postpartum on health, production, and reproductive performance. While these epidemiological studies showed an association and also some evidence for a causal relationship between hypocalcemia and an increased risk for infectious diseases (Martinez et al., 2014), longer risk periods have to be evaluated with caution. It is also possible that reduced feed intake affects serum calcium levels before clinical signs of disease become apparent as most recently shown by Pinedo et al. (2017) for cows suffering from puerperal metritis. In contrast to the physiologically well established risk period of 24 h after parturition, considering a relatively long risk period of 1 week might lead to an underestimation of the prevalence and an overestimation of the effect size caused by hypocalcemia.

Therefore, the objective of this cross-sectional study was to evaluate the impact of hypocalcemia within 48 h after parturition on milk yield, culling, and reproductive performance across different regions and management systems in Germany.

4.4   Materials and methods

4.4.1   Study Population

A cross-sectional study was conducted based on a convenience sample of 125 dairy herds from 8 federal states of Germany between February 2015 and August 2016. Inclusion criteria for herds were 1) participation in a federal DHIA equivalent testing system, 2) freestall housing with at least 100 milking cows, 3) feeding of a TMR-based diet, and 4) a computerized herd management software. Average herd size was 513 and ranged from 112 to 2,607 lactating cows. The average milk production (305d ECM, 4.0 % fat, 3.4 % protein) was 9,231 kg (range 6,257 kg – 10,880 kg). Holstein Frisian cows were the predominant breed on 122 farms. Two farms kept Simmental cattle and one farm kept Jersey as the main breed.

4.4.2 Experimental Procedures

Veterinary practitioners had been invited to participate in the study by an information leaflet sent out by regular mail. Participating practices were informed about the nature and duration of the study and received a package containing blood serum collection systems (S-Monovette 9ml Z, Sarstedt AG & Co, Nürnbrecht, Germany), cryo-vials (Cryvial, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to store serum at -20°C until analysis, and a written standard operating procedure (**SOP**). This SOP described which information to record for each cow enrolled and how to examine the cow before blood collection. A case report form for each cow was provided to document time of sampling, ear tag number, time of calving, calving ease (i.e., unassisted calving or assisted calving with at least one person), clinical symptoms of milk fever (i.e., recumbency), and parity. Administration of calcium products, time relative to calving, and route of administration (i.e., subcutaneous, intravenous, oral) of these products was also documented. Cows with missing information were excluded from analyses (n = 175).

Animals were enrolled by convenience when a veterinarian visited the farm on a given day and an animal met the inclusion criteria of being within 48 h after parturition. For each herd, a backup of the management software was requested 150 days after the last cow was sampled. Herds that did not provide a record were excluded from analyses (n = 9).

4.4.3 Blood Sampling and Laboratory Analyses

Blood samples were taken from the coccygeal vessels using a vacuum blood serum collection system. According to the SOP, samples were kept at room temperature and allowed to clot. Within 5 h of blood collection, samples were centrifuged to harvest serum, which was frozen at -20 °C. Analysis of blood samples was carried out by a commercial laboratory (Synlab Services GmbH, Augsburg, Germany; accreditation number D-PL-14016-01-00 according to the European regulation (EG) No. 765/2008). Total serum calcium concentration was analyzed using photometry (AU680, Beckman Coulter, Krefeld, Germany). The inter- and intraassay coefficient of variation was 1.03 % (Ca 2.37 mmol/l; n = 16) and 1.19 % (Ca 2.40 mmol/l; n = 10), respectively.

4.4.4 Early Lactation Milk Yield

All herds participated in a federal DHIA equivalent testing system. Data collection included date of the test day, DIM at the test day, milk yield (kg/ d), milk fat percentage, milk

protein percentage and somatic cell count. For analysis of the early lactation milk yield, the results of the first three test days were evaluated.

To evaluate the impact of somatic cell count on milk yield, the linear score was used. Therefore, the somatic cell count was log transformed as $[\ln(SCC/100)/\ln(2)] + 3$ (Ali and Shook, 1980).

4.4.5 Reproductive Management

Inseminations and pregnancy diagnosis were recorded until 150 DIM. Pregnancy diagnosis was performed by transrectal palpation or ultrasonography between 32 and 45 d after parturition. Reproductive performance was evaluated by DIM at first service, first service conception risk (**FSCR**), and time to pregnancy within 150 DIM.

4.4.6 Postpartum Culling

Culling events were collected from enrollment in the study until 60 DIM. Cows were considered to be culled during the postpartum period if removed from the herd at ≤ 60 DIM, except for sales.

4.4.7 Statistical Analyses

Individual cow data were transferred to Microsoft Excel (Office 2013, Microsoft Deutschland Ltd., Munich, Germany). Statistical analyses were performed using SPSS for Windows (version 22.0, SPSS Inc., IBM, Ehningen, Germany).

Appropriate thresholds for defining hypocalcemia based on serum calcium levels associated with an increased risk of negative downstream events were first determined by creating incremental thresholds of 0.1 mmol/L of calcium from 1.8 to 2.2 mmol/L. These thresholds were evaluated using dichotomous variables, designating a 1 value for all samples below each threshold and assigning a value of 0 to all values at or above each threshold. Based on the quality of data (e.g. continuous, categorical, and time to event) for these negative downstream events, we used three different approaches. In all cases, thresholds of calcium were identified from serum obtained from 0 to 48 h postpartum.

Approach 1 – Continuous data. To identify a threshold associated with a change in milk production, we used the following approach. A data set was created from information of

the first 3 test days and serum calcium levels. Hierarchical dummy variables were created in the same manner as described above and ranged from 1.8 to 2.2 mmol/L of serum calcium concentration. To evaluate the effect of calcium status (i.e., normocalcemia vs. hypocalcemia) on milk yield across the first 3 test days, repeated measures ANOVA with first-order autoregressive covariance was performed using the GENLINMIXED procedure of SPSS. The outcome variable was milk yield (kg/d). Cow was the experimental unit and herd was considered as a random effect. Each dummy variable for serum calcium was offered to the model separately. The calcium threshold for the dummy variable having the most extreme (negative or positive) β coefficient and the smallest *P*-value was chosen as the threshold for defining hypocalcemia. Choosing this threshold helps to minimize the chance of a type 1 error. According to the model-building strategies described by Dohoo et al. (2009), each parameter considered for the mixed model was separately analyzed in a univariate model, including the parameter as a fixed factor (i.e., categorical parameter) or covariate (i.e., continuous parameter). Only parameters resulting in univariate models with $P \leq 0.2$ were included in the final mixed model. Selection of the model that best fit the data was performed by testing each effect separately in a multivariable univariate model and finding the model with the lowest value for the Akaike information criterion (AIC) using a backward elimination procedure that removed all variables with $P > 0.10$ from the model. Regardless of the significance level, calcium status was forced to remain in the model. We used 2 separate models for primiparous and multiparous animals to account for their marked difference in milk yield and in order to include previous lactation milk as a covariate for multiparous cows. The initial model for primiparous cows contained the following explanatory variables as fixed effects: breed (Holstein, Jersey or Simmental), somatic cell count (continuous), oral calcium supplementation (yes vs. no), time of test day (first, second or third test day after parturition), calving ease (unassisted calving vs. assisted calving) and calcium status 0 – 48 h after parturition (normocalcemia vs. hypocalcemia). The initial model for multiparous cows contained the same explanatory variables as for primiparous cows. Additionally, the 305 d milk yield of the previous lactation (continuous), milk fever (yes vs. no), and parity (lactation 2; 3; or 4+) was considered for multiparous cows.

Approach 2 – Categorical data. Using failure of conception at first AI as the gold standard, sensitivity and specificity for each threshold was calculated (Table 6). The serum calcium threshold having the greatest sum of sensitivity and specificity was selected for further logistic regression analysis. To further evaluate the effect of calcium status on FSCR, a logistic regression model using the GENLINMIXED procedure of SPSS was built. Cow was the experimental unit. Herd was considered as a random effect. Model building was conducted as recommended by Dohoo et al. 2009, where each parameter was first analyzed separately in a univariate model including the parameter as a fixed factor (i.e., categorical parameter) or

covariate (i.e., continuous parameter). Only parameters resulting in univariate models with *P* ≤ 0.2 were included in the final mixed model. Selection of the model that best fit the data was performed by testing each effect separately in a multivariable univariate model and finding the model with the lowest value for the Akaike information criterion (AIC) using a backward elimination procedure that removed all variables with *P* > 0.20 from the model. The initial model contained the following explanatory variables as fixed effects: parity (lactation 1; 2; 3; or 4+), breed (Holstein, Jersey or Simmental), calving ease (unassisted calving vs. assisted calving), DIM at first AI (continuous), oral calcium supplementation (yes vs. no), calcium status (normocalcemia; hypocalcemia). Regardless of the significance level, type of calcium status was forced to remain in the model.

Approach 3 – Time to event data. Shared frailty models were built to predict time to first insemination and time to pregnancy within the first 150 DIM and time to culling within the first 60 DIM using penalized log likelihood method. Herd was used as a frailty effect to account for clustering of observations within herds. Cows were censored if they were culled before first insemination or pregnancy, respectively, or at the end of the observation period. The models were constructed for each of the five cut-offs (1.8, 1.9, 2.0, 2.1, 2.2 mmol/L) for serum calcium concentration. The variables oral calcium supplementation, breed, parity, milk fever, and assisted calving or a combination thereof were tested as risk factors. Models with the most extreme ß-value for hypocalcemia and the lowest *P*-value were selected. Proportional hazards assumption was graphically assessed. To evaluate the effect of clustering by herd, we provided the theta values. Large theta values indicate a major influence of herd on the parameter of interest. Frailty models were built using the computer program Stata (Stata/IC 13.1 for Windows, StataCorp LP, Texas, US).

4.5   Results

Overall, blood samples were drawn from 1,709 animals at 0 to 48 h after calving from 125 farms. Nine farms with 108 animals were excluded from analyses because they did not provide a data backup. Further 175 animals were excluded because their case reports were incomplete or incorrect (Figure 1).

Data of 1,426 animals (83.4 %) were available for final analyses. Of those, 211 (14.8 %), 384 (26.9 %), 354 (24.8 %), and 477 (33.5 %) were in first, second, third, and ≥ fourth lactation, respectively.

4.5.1   Milk Yield

Primiparous Cows. Animals that suffered from subclinical hypocalcemia (threshold: 2.0 mmol/L) tended to produce less milk than normocalcemic cows in early lactation (- 2.07 kg/d; *P* = 0.063). Considering the somatic cell count, cows produced 0.52 kg/d less milk for every one unit increase of the linear score (*P* = 0.001). Primiparous Jersey cows produced less milk than Holstein primiparous cows (7.84 kg/d; *P* = 0.034). Results for primiparous cows are summarized in Table 1 and 2.

Multiparous Cows. Cows suffering from clinical milk fever produced less milk than normocalcemic cows in early lactation (- 1.9 kg/d; *P* = 0.002; Table 4). There was a tendency for hypocalcemic cows (threshold 2.1 mmol/L; Table 3) to produce 0.51 kg/d more milk than normocalcemic cows (*P* = 0.086; Table 4). For every one unit increase of the linear score, multiparous cows produced 0.71 kg/d less milk than healthy cows in early lactation (*P* = 0.001; Table 4). Multiparous Jersey cows produced 7.08 kg/d less milk than Holstein cows (*P* = 0.016; Table 4). Cows with an assisted calving had a lower milk yield in early lactation than cows with an unassisted calving (- 1.10 kg/d; *P* = 0.010; Table 4). Moreover, milk production level in previous lactation positively affected milk production level in the subsequent lactation (+ 1.49 kg/d; *P* = 0.001; Table 4).

4.5.2 Reproductive Performance

Days to First Insemination. We were not able to identify a threshold for hypocalcemia with a significant effect (*P* > 0.05; Table 5, Figure 2) on days to first insemination. Median days to first insemination were 71 and 73 for normocalcemic and hypocalcemic animals, respectively. Parity affected days to first insemination. Cows in the first and second lactation had greater hazards of being inseminated per time interval compared to cows in higher lactations. The lowest hazard of being inseminated was found for cows in the fourth and higher lactation. Theta was 0.306 with a SE of 0.062 (*P* < 0.001), indicating that the herd had a significant influence on the day when a cow was first inseminated.

First Service Conception Risk. Cows with hypocalcemia (threshold 1.9 mmol/L) had decreased odds (odds ratio [**OR**] = 0.56; *P* = 0.001; Table 7) of pregnancy at first AI. There was no effect of clinical milk fever on FSCR. Cows in third lactation had reduced odds of pregnancy at first AI compared to primiparous cows (OR = 0.61; *P* = 0.026).

Time to Pregnancy. Calcium status had a significant influence on time to pregnancy (Table 8; Figure 3). Cows with hypocalcemia (threshold: 1.8 mmol/L) had a significantly reduced hazard of pregnancy compared with normocalcemic cows (hazard ratio [**HR**] = 0.69, *P* = 0.001). Median time to pregnancy was 109 and 134 days for normocalcemic and

hypocalcemic animals, respectively. Moreover, cows in the third (HR = 0.74; *P* = 0.019) and fourth and higher lactation (HR = 0.76; *P* = 0.029) had a significantly reduced hazard of getting pregnant compared with cows in the first lactation. Theta was 0.286 (SE 0.063; *P* < 0.001) showing a significant effect of the herd on time to pregnancy.

4.5.3 Early Lactation Culling

Cows with subclinical hypocalcemia (threshold: 2.0 mmol/L) had a 1.6 times greater hazard of being culled (*P* < 0.013; Figure 4; Table 9) in early lactation. Cows with milk fever had a 1.9 times greater hazard of being culled within the first 60 DIM compared with normocalcemic cows (*P* = 0.050). Significant theta values of 0.656 (SE = 0.245; *P* < 0.001) indicate a clustering effect by herd.
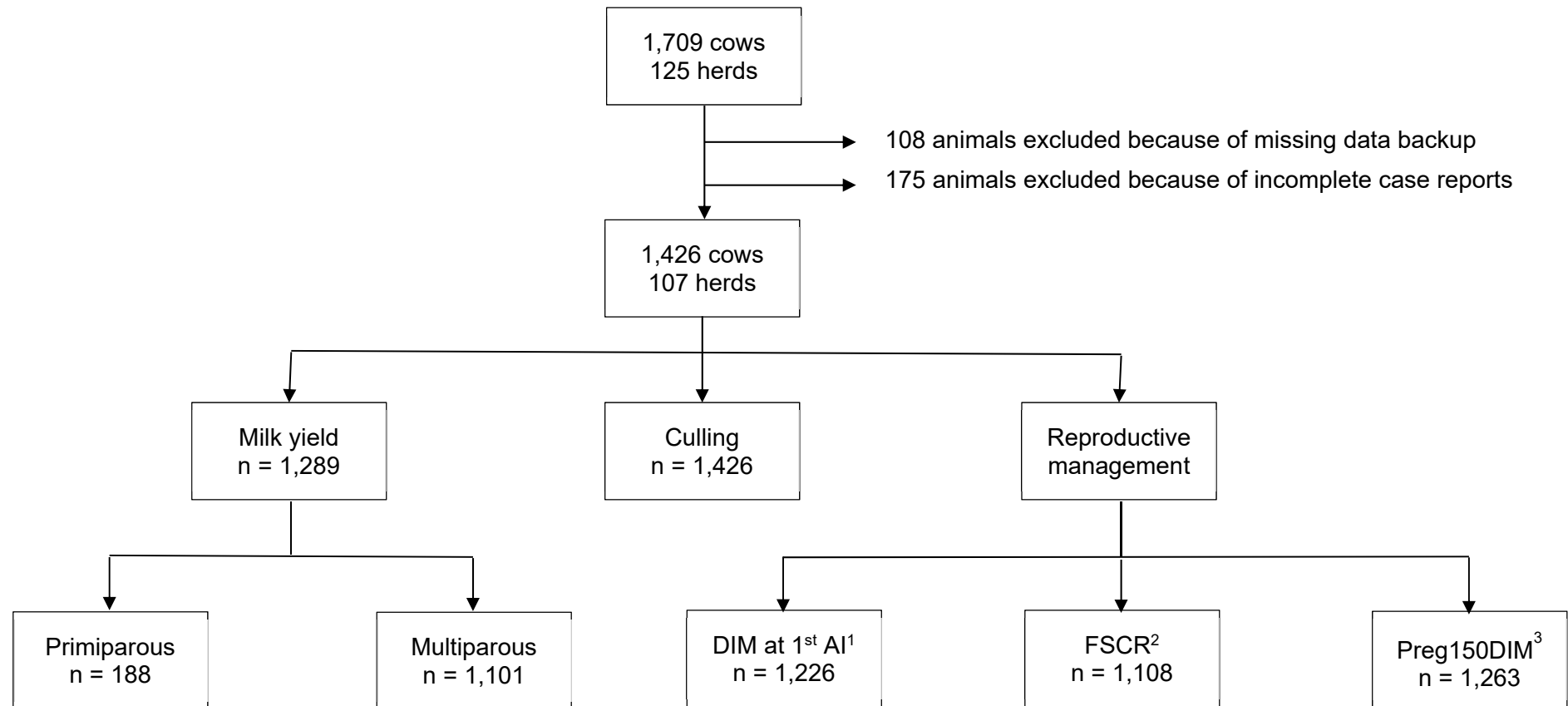
Additional unpublished work

Figure 1. Flowchart of total number of animals screened for hypocalcemia and considered for final analyses. Number of observations included in each analysis might differ due to several reasons (e.g., culling, do not breed decision, missing test day information).

[1] Days to first insemination within 150 DIM.

[2] First service conception risk

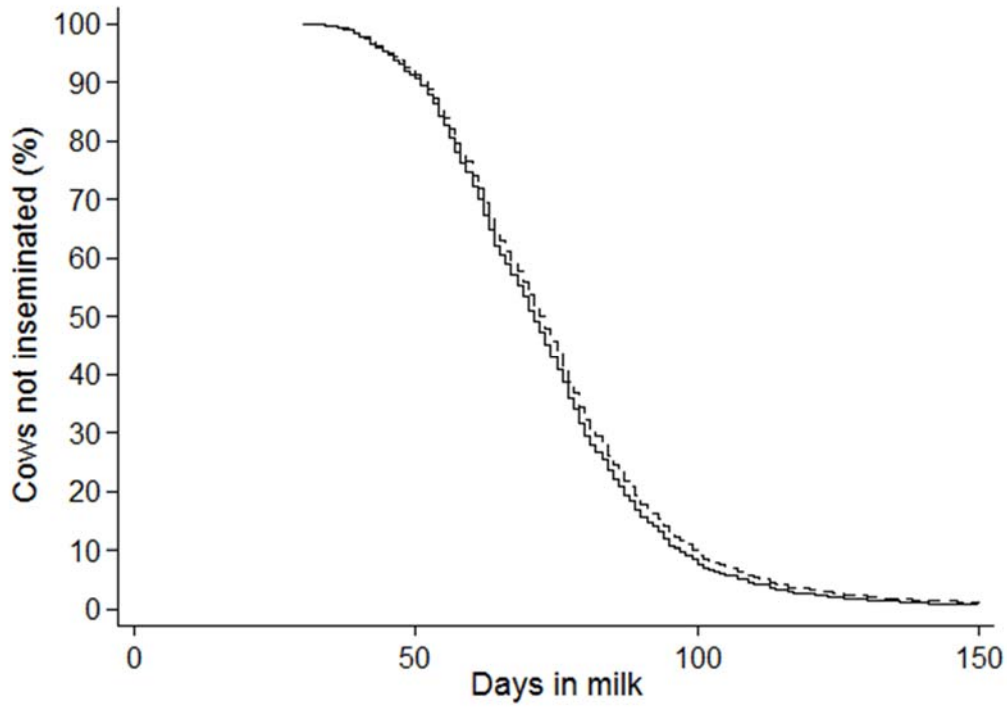[3] Time to first insemination within 150 DIM

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Figure 2. Cox proportional hazards regression with shared frailty effects for herds illustrating the effect of calcium status on time to first artificial insemination within the first 150 days in milk (normocalcemia = solid curve, Ca ≥ 2.2 mmol/L; hypocalcemia = dashed curve, Ca < 2.2 mmol/L). Cows were censored if they were culled or at the end of the observation period.
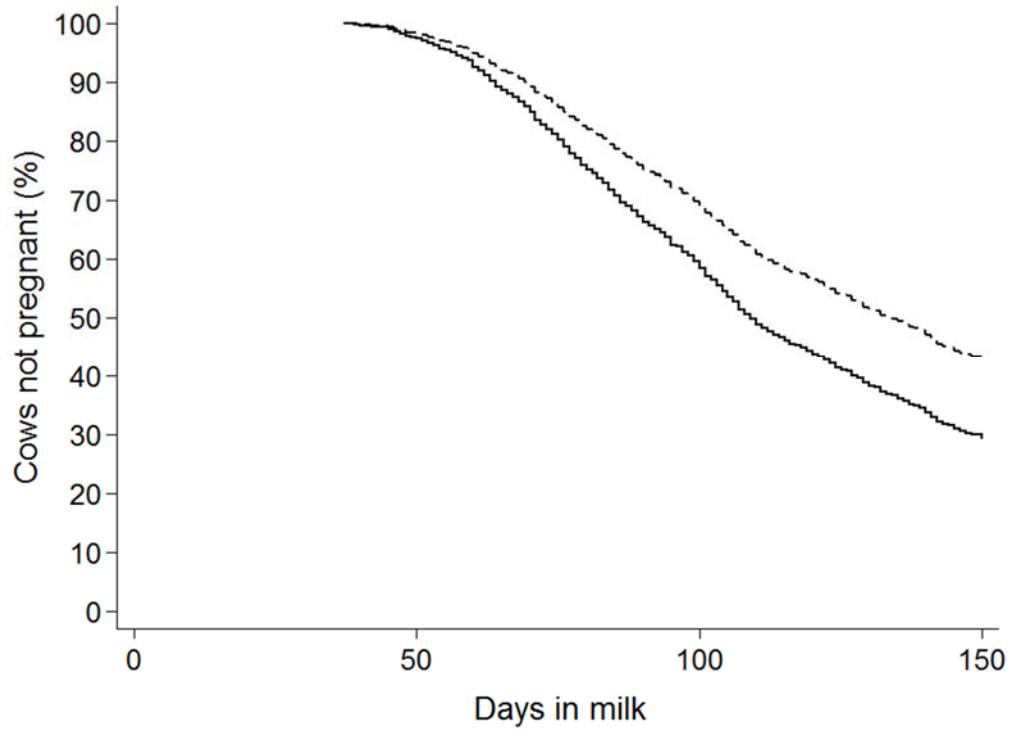
Figure 3. Cox proportional hazards regression with shared frailty effects for herds illustrating the effect of calcium status on time to pregnancy within the first 150 days in milk (normocalcemia = solid line, Ca ≥ 1.8 mmol/L; hypocalcemia = dashed line, Ca < 1.8 mmol/L). Cows were censored if they were culled or at the end of the observation period.
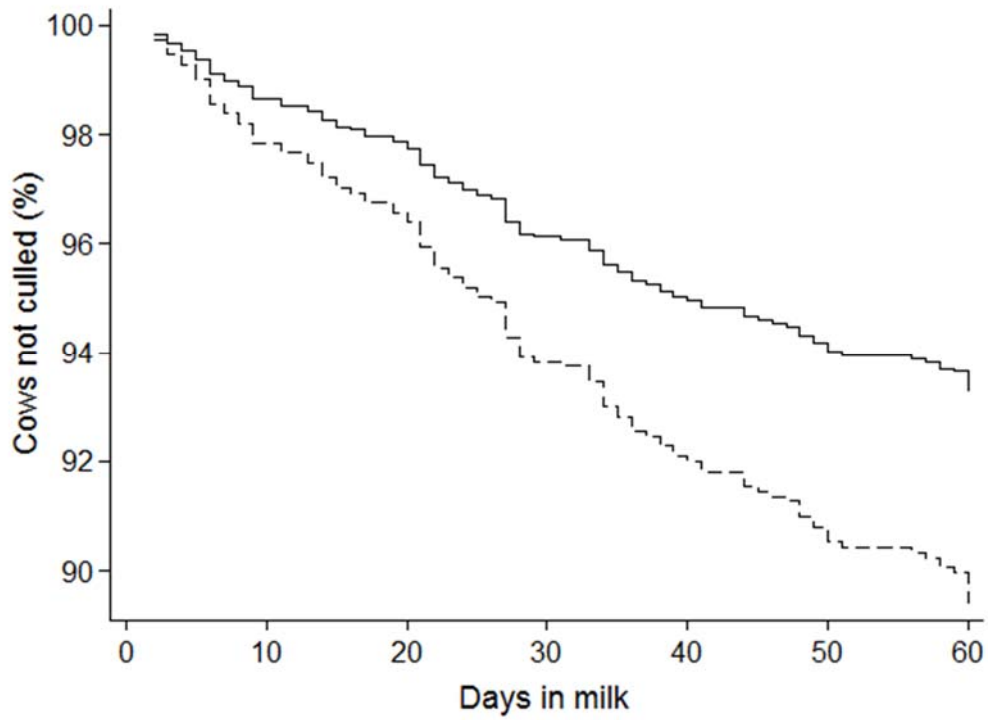
Figure 4. Cox proportional hazards regression with shared frailty effects for herds illustrating the effect of calcium status on culling within the first 60 DIM (normocalcemia = solid line; Ca ≥ 2.0 mmol/L; hypocalcemia = dashed line; Ca < 2.0 mmol/L).

Table 1. The impact of decreased serum calcium concentration 0 to 48 h after parturition on milk yield[1] of primiparous cows across the first three test days.

Threshold calcium (mmol/L)	Milk yield for cows at or above threshold (kg)	Milk yield for cows below threshold (kg)	Difference in yield for cows at or above threshold (kg)	*P*-value
1.8	26.57	26.45	-0.12	0.953
1.9	26.58	24.43	-2.15	0.253
2.0*	26.60	24.54	-2.06	0.063
2.1	26.62	25.89	-0.73	0.330
2.2	26.80	25.96	-0.84	0.130

[1]Least squares means derived from a mixed model. Model adjusted for the random effect of herd and the fixed effects of breed, test day, and SCC linear score using GENLINMIXED procedure in SPSS version 22.0.

*Optimum threshold based on combination of least *P*-value and greatest magnitude of effect indicated with an asterisk.

Table 2. Effect of hypocalcemia within 48 h after parturition on milk production across the first 3 test days for 188 primiparous dairy cows.

Variable[1]	Estimate milk (kg)	SE	95 % CI		P-value
			Lower CI	Upper CI	
Intercept	31.53	1.15	29.28	33.78	0.001
Breed					
Holstein	Referent				
Simmental	-4.49	2.73	-9.86	0.88	0.101
Jersey	-7.84	3.70	-15.10	-0.59	0.034
Linear score	-0.52	0.16	-0.84	-0.21	0.001
Test day					
1st test day	Referent				
2nd test day	2.73	0.53	1.68	3.78	0.001
3rd test day	4.02	0.60	2.84	5.19	0.001
Calcium status[2]					
Normocalcemia	Referent				
Hypocalcemia	-2.07	1.11	-4.25	0.11	0.063

[1] Model adjusted for the random effect of herd.

[2] Calcium status: normocalcemia was defined as serum calcium concentration greater or equal to 2.0 mmol/L. Cows with a serum calcium concentration below 2.0 mmol/L were categorized as hypocalcemic animals.

Table 3. The impact of decreased serum calcium concentration 0 to 48 h after parturition on milk yield[1] of multiparous cows across the first three test days.

Threshold calcium (mmol/L)	Milk yield for cows at or above threshold (kg)	Milk yield for cows below threshold (kg)	Difference in yield for cows at or above threshold (kg)	*P*-value
1.8	36.44	36.37	-0.07	0.830
1.9	36.18	36.59	+0.41	0.177
2.0	36.37	36.57	+0.20	0.487
2.1*	36.07	36.59	+0.52	0.086
2.2	35.90	36.46	+0.56	0.092

[1]Least squares means derived from a mixed model. Model adjusted for the random effect of herd and the fixed effects of breed, test day, previous lactation milk yield, SCC linear score, recumbency, and calving ease, using GENLINMIXED procedure in SPSS version 22.0.

*Optimum threshold based on combination of least *P*-value and greatest magnitude of effect indicated with an asterisk.

Table 4. Effect of hypocalcemia within 48 h after parturition on milk production across the first 3 test days for 1,101 multiparous dairy cows.

Variable[1]	Estimate milk (kg)	SE	95 % CI		*P*-value
			Lower CI	Upper CI	
Intercept	26.02	0.88	24.30	27.73	0.001
Breed					
Holstein	Referent				
Simmental	0.18	2.30	-4.33	4.68	0.939
Jersey	-7.08	2.95	-12.85	-1.30	0.016
Linear score	-0.71	0.07	-0.84	-0.58	0.001
Test day					
1st test day	Referent				
2nd test day	3.46	0.32	2.84	4.09	0.001
3rd test day	1.37	0.33	0.72	2.03	0.001
Calving ease					
Unassisted	Referent				
Assisted	-1.10	0.43	-1,93	-0.26	0.010
Previous lactation milk[2]	1.47	0.08	1.31	1.62	0.001
Calcium status[3]					
Normocalcemia	Referent				
Hypocalcemia	0.51	0.30	-0.07	1.10	0.086
Clinical milk fever[4]					
No	Referent				
Yes	-1.90	0.62	-3.11	-0.68	0.002

[1] Model adjusted for the random effect of herd.

[2] Previous lactation milk: 305d milk yield (kg) of the previous lactation.

[3] Calcium status: normocalcemia was defined as serum calcium concentration greater or equal to 2.1 mmol/L. Optimum threshold was chosen based on the combination of least *P*-value and the greatest magnitude of effect of hypocalcemia on milk yield for the first 3 test days.

[4] Clinical milk fever: recumbent cows with a serum calcium concentration below 2.1 mmol/L were defined as cows suffering from clinical milk fever.

Table 5. Cox regression model with shared frailty effects for herds predicting days to first insemination within 150 DIM (n = 1,108).

Variable	HR[1]	SE	95 % CI		P-value
			Lower CI	Upper CI	
Calcium status[2]					
Normocalcemia	Referent				
Hypocalcemia	0.93	0.07	0.80	1.07	0.296
Parity					
Lactation 1	Referent				
Lactation 2	1.11	0.12	0.90	1.37	0.317
Lactation 3	0.88	0.10	0.71	1.09	0.238
Lactation 4+	0.82	0.09	0.67	1.01	0.060

Note: Standard errors of hazard ratios are conditional on theta.

Cows were censored if they were culled before being inseminated or at 150 DIM.

[1] Hazard ratio; HR for being inseminated within 150 DIM. HR < 1 indicates a reduced instantaneous relative risk of insemination.

[2] Calcium status: normocalcemia was defined as serum calcium concentration greater or equal to 2.2 mmol/L. Cows with a serum calcium concentration below 2.2 mmol/L were categorized as hypocalcemic animals.

Table 6. Evaluation of the risk to get pregnant at first artificial insemination and univariate associations with serum calcium concentration obtained 0 to 48 h after parturition in 1,056 cows.

Calcium threshold (mmol/L)	Proportion of cows below threshold	Risk of pregnancy at first AI at or above threshold	Risk of pregnancy at first AI below threshold	Odds ratio[1]	95 % CI	*P*-value	Sensitivity (%)	Specificity (%)
1.8	24.0	40.3	29.8	1.59	1.18 – 2.14	0.002	27.1	81.0
1.9*	32.9	41.5	29.9	1.66	1.27 – 2.17	0.001	37.0	73.9
2.0	40.8	41.6	32.3	1.49	1.16 – 1.91	0.002	44.4	65.2
2.1	54.1	41.1	35.0	1.30	1.02 – 1.65	0.036	56.6	49.9
2.2	69.3	42.9	35.6	1.36	1.05 – 1.76	0.021	71.8	34.8

[1] Odds ratio: odds ratio > 1.0 indicating a higher risk for non-pregnancy at first AI for cows with hypocalcemia.

*Optimum threshold based on maximum sensitivity and specificity.

Table 7. Final multivariable logistic regression model evaluating the effect of calcium status on pregnancy per AI at first AI in 1,108 dairy cows.

Variable[1]	Estimate	SE	95 % CI		Odds ratio	*P*-value
			Lower CI	Upper CI		
Intercept	-0.97	0.28	-1.52	-0.42	0.38	0.001
Parity						
Lactation 1	Referent					
Lactation 2	-0.22	0.21	-0.63	0.19	0.80	0.291
Lactation 3	-0.50	0.23	-0.94	-0.06	0.61	0.026
Lactation 4+	-0.29	0.22	-0.72	0.14	0.75	0.186
DIM at 1st AI	0.01	0.00	0.01	0.02	1.01	0.001
Calcium status[2]						
Normocalcemia	Referent					
Hypocalcemia	-0.57	0.16	-0.89	-0.26	0.56	0.001

[1] Model adjusted for the random effect of herd.

[2] Calcium status: normocalcemia was defined as serum calcium concentration greater or equal to 1.9 mmol/L. Cows with a serum calcium concentration below 1.9 mmol/L were categorized as hypocalcemic animals.

Table 8. Cox regression model with shared frailty effects for herds predicting DIM to pregnancy within 150 DIM (n = 1,263).

Variable	HR[1]	SE	95 % CI		P-value
			Lower CI	Upper CI	
Calcium status[2]					
Normocalcemia	Referent				
Hypocalcemia	0.69	0.07	0.57	0.85	<0.001
Parity					
Lactation 1	Referent				
Lactation 2	1.00	0.12	0.79	1.27	0.999
Lactation 3	0.74	0.10	0.58	0.95	0.019
Lactation 4+	0.76	0.09	0.60	0.97	0.029

Note: Standard errors of hazard ratios are conditional on theta.

Cows were censored if they were culled before being pregnant or at 150 DIM.

[1] Hazard ratio; HR for becoming pregnant within 150 DIM. HR < 1 indicates a reduced instantaneous relative risk of pregnancy within 150 DIM.

[2] Calcium status: normocalcemia was defined as serum calcium concentration greater or equal to 1.8 mmol/L. Cows with a serum calcium concentration below 1.8 mmol/L were categorized as hypocalcemic animals.

Table 9. Cox regression model with shared frailty effects for herds predicting culling within 60 DIM (n = 1,426).

Variable	HR[1]	SE	95 % CI		*P*-value
			Lower CI	Upper CI	
Calcium status[2]					
Normocalcemia	Referent				
Hypocalcemia	1.61	0.31	1.10	2.35	0.013
Clinical milk fever[3]					
No	Referent				
Yes	1.90	0.32	1.00	3.60	0.050

Note: Standard errors of hazard ratios are conditional on theta.

Cows were censored at 60 DIM.

[1] Hazard ratio; HR for culling within 60 DIM. HR < 1 indicates a reduced instantaneous relative risk of culling within 60 DIM.

[2] Calcium status: normocalcemia was defined as serum calcium concentration greater or equal to 2.0 mmol/L. Cows with a serum calcium concentration below 2.0 mmol/L were categorized as hypocalcemic animals.

[3] Clinical milk fever: recumbent cows with a serum calcium concentration below 2.0 mmol/L were defined as cows suffering from clinical milk fever.

4.6   Discussion

As recently reported, prevalence of clinical and subclinical hypocalcemia around parturition is high in German dairy herds and most dairies did not implement a suitable preventive strategy (Venjakob et al., 2017). The results of the present study underline that hypocalcemia has negative consequences for early lactation milk yield, culling, and reproductive performance. Different thresholds for serum calcium concentration 0 to 48 h after parturition were identified for these outcomes (2.0 and 2.1 mmol/L for milk yield of primiparous and multiparous cows, respectively; 1.9 mmol/L for FSCR; 1.8 mmol/L for time to pregnancy, and 2.0 mmol/L for culling).

4.6.1   Milk Production

There was a tendency (P = 0.063) for primiparous cows suffering from hypocalcemia (< 2.0 mmol/L) to produce 2.07 kg/d less milk compared with normocalcemic primiparous cows in early lactation. On the other hand, multiparous cows affected by hypocalcemia (< 2.1 mmol/L) had a tendency towards higher milk yield (+ 0.52 kg/d) in early lactation. In contrast, Chapinal et al. (2012a) found an association of hypocalcemia (< 2.1 mmol/L) one week pre- and up to three weeks postpartum with decreased milk yield. Serum calcium concentration ≤ 2.1 mmol/L one week before und two and three weeks after parturition was associated with -3.2, -2.6, and -3.5 kg milk per day across the first four DHIA tests in lactation, respectively. Serum calcium concentration ≤ 2.1 mmol/L in week one, two and three postpartum were associated with -2.6, -4.8 and -7.1 kg/d milk only at first DHIA test day, respectively, irrespective of parity. The authors speculated that the observed low calcium values might also be an indicator of inadequate feed intake rather than metabolic disease when considering such a long risk period. This assumption is in agreement with Pinedo et al. (2017), who suggested that hypocalcemia might be caused by insufficient feed intake, prior to clinical signs of postpartum diseases. Using a matched pair comparison, Jawor et al. (2012) detected greater milk yield (+ 6 kg/d) during week 2, 3, and, 4 postpartum for cows suffering from subclinical hypocalcemia 0 to 24 h after calving (< 1.8 mmol/L). These results are supported by a more recent study that found greater milk yield in cows suffering from subclinical hypocalcemia on the first, second and third DHIA equivalent test postpartum (Gild et al., 2015). This is in agreement with our data that show a tendency in multiparous cows with hypocalcemia to produce 0.5 kg more milk in early lactation using 2.1 mmol/L as a threshold. However, another study described that in primiparous and multiparous cows, milk yield did not differ between normocalcemic and hypocalcemic animals (Martinez et al., 2012).

None of the aforementioned studies took previous lactation milk yield into account. By ignoring previous lactation milk yield, the association between hypocalcemia and milk yield might be biased. It has been shown that cows with high milk yield in the previous lactation will produce more milk in the subsequent lactation (Nordlund, 2006). However, high milk yield in previous lactation is also an important risk factor for hypocalcemia (Fleischer et al., 2001).

Furthermore, our study demonstrated that multiparous cows with clinical milk fever produced 1.9 kg/d less milk during the first three months of lactation. Two previous studies (Rajala-Schultz et al., 1999; Fleischer et al., 2001) based on 20,983 Finish Ayrshire and 1,074 Holstein Frisian cows, respectively failed to demonstrate an impact of clinical milk fever on milk yield, when compared with normocalcemic cows. Data from these studies originate from two decades ago and the latter study reported an average 305-d milk yield of 7,802 kg. We speculate that today's dairy cows suffering from milk fever were not able to compensate the loss of milk caused by recumbency in early lactation. In contrast to the subclinical form, clinical milk fever is a more severe state of maladaptation, therefore affecting milk yield negatively.

Using the linear score to analyze the impact of high somatic cell count on milk yield, milk production decreased by 0.52 and 0.72 kg/d for every one unit increase of somatic cell count in primiparous and multiparous cows, respectively. High somatic cell count is an indicator for poor udder health and can be a symptom of subclinical or clinical mastitis (Harmon, 1994). Gröhn et al. (2004) showed that milk yield began to drop already two to one week before diagnosis of mastitis. The greatest loss occurred directly after diagnosis and often milk production did not recover to the potential yield.

Primiparous and multiparous Jersey cows produced significantly less milk than Holstein cows (- 7.84 kg/d; *P* = 0.034; Table 2 and - 6.99 kg/d; *P* = 0.017; Table 4). This is in agreement with a report in which breed (Jersey cows produced 23.3 % less milk compared to Holstein cows) had a significant effect on milk yield (White et al., 2002). Considering milk yield of breeds, our results should be interpreted with caution, because only one of the participating dairy farms kept Jersey cows as predominant breed. Also, herd management might have biased the impact of breed on milk production.

Our study re-emphasizes the importance of a sound calving management. Dystocia had a strong negative effect on milk production. Cows with unassisted calving produced 1.1 kg/d more milk in early lactation compared to cows with assisted calving. This has previously been shown by Dematawewa and Berger (1997), who analysed records of 71,618 US Holstein cows. Discriminating between various severities of dystocia (5-point scale), there was a decrease of 305-d milk yield with increasing severity of dystocia. Other authors found an effect

of caesarean section on milk production at first DHIA equivalent, but no effect of mild and severe dystocia on milk yield (Tenhagen et al., 2007).

4.6.2 Reproductive Management

Cows suffering from hypocalcemia had decreased odds (OR 0.56; *P* = 0.001) of becoming pregnant at first AI. In a recent study, Caixeta et al. (2017) investigated the impact of hypocalcemia on estrus cyclicity and FSCR. The authors took blood samples on the first three DIM and cows with a serum calcium concentration above 2.15 mmol/L were considered normocalcemic. Subclinical hypocalcemia was defined as serum calcium concentration ≤ 2.15 mmol/L on one of the first three DIM while chronic subclinical hypocalcemia was defined as serum calcium concentration ≤ 2.15 mmol/L for all three DIM measured. Return to cyclicity took longer for cows with chronic subclinical hypocalcemia, compared with normocalcemic animals and animals with subclinical hypocalcemia. Moreover, cows with chronic subclinical hypocalcemia had decreased odds of becoming pregnant at first AI.

Our data confirm these observations, which also showed that cows with subclinical hypocalcemia had a significantly reduced hazard of getting pregnant within 150 DIM. On average, it took 15 d longer for cows with hypocalcemia to get pregnant compared with normocalcemic cows. This is in agreement with Martinez et al. (2012), who found a tendency for normocalcemic animals to have a greater risk of pregnancy in 230 DIM compared with hypocalcemic cows. Cows suffering from a clinical or subclinical disease (e.g. subclinical hypocalcemia) were described to have a reduced probability of being cyclic, which might be an explanation for reduced pregnancy among hypocalcemic cows (Ribeiro et al., 2013). In comparison, Gild et al. (2015) did not find a difference in cumulative conception until 180 DIM between normocalcemic and hypocalcemic animals.

Cows being in third or ≥ fourth lactation had a significantly reduced hazard of getting pregnant in comparison to primiparous cows; a finding that is well known in the literature (Carvalho et al., 2015; Fuenzalida et al., 2015). Furthermore, multiparous cows have a higher risk for pregnancy loss (Galvao et al., 2007; Carvalho et al., 2015).

Cows with an assisted calving had a tendency towards a delayed time of pregnancy. This is in agreement with previous studies showing that cows with severe dystocia conceived later and were more likely to be open by 200 DIM (Tenhagen et al., 2007).

4.6.3 Culling

Cows with hypocalcemia had a 1.6 greater hazard of being culled during the first 60 DIM. Other authors have reported similar results about the impact of hypocalcemia on early lactation culling. These studies, however, considered longer risk periods for cows to be hypocalcemic. Seifi et al. (2011) found 2.4 times and 5.3 greater odds for cows to get culled when serum calcium concentration was ≤ 2.2 mmol/L one week and ≤ 2.3 mmol/L in week two postpartum, respectively. Further evidence was provided by Roberts et al. (2012) who described that serum calcium concentrations ≤ 2.2 and ≤ 2.3 mmol/L in week one and two after calving, respectively, were associated with an increased culling risk.

Furthermore, in our study, cows with milk fever had a 1.9 times greater hazard of being culled within the first 60 DIM compared with normocalcemic cows. This finding supports early evidence by Gröhn et al. (1998) that cows with clinical milk fever are more than twice as likely to get culled, compared with healthy cows.

4.7   Study Limitations

Due to the nature of a large multi-centric study design and the sampling scheme of one blood sample 0 to 48 h after calving, we were not able to discriminate between acute and chronic hypocalcemia. It seems likely that cows exposed to hypocalcemia for a longer period are predominantly affected by downstream outcomes (e.g., delayed return to cyclicity, reduced FSCR) as recently shown by Caixeta et al. (2017).

The statistical method to determine critical thresholds for hypocalcemia was based on the nature of the outcome (i.e., binary, continuous, time to event). To account for the effect of clustering of cows within herds, we favored the use of either a generalized linear mixed model or frailty models. The same methods were used before to establish thresholds for BHBA (Duffield et al., 2009). By using dummy variables, we tested calcium thresholds from 1.8 mmol/L to 2.2 mmol/L. Showing an association with negative health or production outcomes in recent literature (Seifi et al., 2011; Chapinal et al., 2012b; Jawor et al., 2012; Martinez et al., 2012), this range of threshold appeared plausible.

4.8   Conclusions

Overall, our multicentric study including 1,426 cows from 115 farms shows that hypocalcemia 0 to 48 h after calving had a negative impact on reproduction and culling risk.

Cows suffering from hypocalcemia had decreased odds of becoming pregnant at first AI and a reduced hazard of becoming pregnant within 150 DIM. Hypocalcemia and clinical milk fever led to a 1.6 and 1.9 greater risk of being culled within the first 60 DIM, respectively. The effect on milk yield was conditional on parity. Primiparous cows suffering from hypocalcemia had a tendency to produce less milk and multiparous cows had a tendency to produce more milk compared with normocalcemic cows. Presence of milk fever had a negative effect on milk yield. Given the high prevalence of hypocalcemia and the associated negative downstream outcomes, this study re-emphasizes the need to establish control strategies for hypocalcemia. The results of this study are based on a large number of herds with different managerial conditions and the established thresholds seem to be applicable to high producing dairy cows within the first 48 h after calving.

4.9   References

Ali, A. K. A. and G. E. Shook. 1980. An optimum transformation of somatic cell concentration in milk. J. Dairy Sci. 63:487-490.

Caixeta, L. S., P. A. Ospina, M. B. Capel, and D. V. Nydam. 2017. Association between subclinical hypocalcemia in the first 3 days of lactation and reproductive performance of dairy cows. Theriogenology 94:1-7.

Carvalho, P. D., M. J. Fuenzalida, A. Ricci, A. H. Souza, R. V. Barletta, M. C. Wiltbank, and P. M. Fricke. 2015. Modifications to Ovsynch improve fertility during resynchronization: Evaluation of presynchronization with gonadotropin-releasing hormone 6 d before initiation of Ovsynch and addition of a second prostaglandin F2alpha treatment. J. Dairy Sci. 98:8741-8752.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. J. Dairy Sci. 94:4897-4903.

Chapinal, N., M. E. Carson, S. J. LeBlanc, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012a. The association of serum metabolites in the transition period with milk production and early-lactation reproductive performance. J. Dairy Sci. 95:1301-1309.

Chapinal, N., S. J. Leblanc, M. E. Carson, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012b. Herd-level association of serum metabolites in the

transition period with disease, milk production, and early lactation reproductive performance. J. Dairy Sci. 95:5676-5682.

DeGaris, P. J. and I. J. Lean. 2008. Milk fever in dairy cows: a review of pathophysiology and control principles. Vet. J. 176:58-69.

Dematawewa, C. M. and P. J. Berger. 1997. Effect of dystocia on yield, fertility, and cow losses and an economic evaluation of dystocia scores for Holsteins. J. Dairy Sci. 80:754-761.

Duffield, T. F., K. D. Lissemore, B. W. McBride, and K. E. Leslie. 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. J. Dairy Sci. 92:571-580.

Fleischer, P., M. Metzner, M. Beyerbach, M. Hoedemaker, and W. Klee. 2001. The relationship between milk yield and the incidence of some diseases in dairy cows. J. Dairy Sci. 84:2025-2035.

Fuenzalida, M. J., P. M. Fricke, and P. L. Ruegg. 2015. The association between occurrence and severity of subclinical and clinical mastitis on pregnancies per artificial insemination at first service of Holstein cows. J. Dairy Sci. 98:3791-3805.

Galvao, K. N., M. F. Sa Filho, and J. E. Santos. 2007. Reducing the interval from presynchronization to initiation of timed artificial insemination improves fertility in dairy cows. J. Dairy Sci. 90:4212-4218.

Gild, C., N. Alpert, and M. van Straten. 2015. The Influence of SCH on Milk Production and Reproduction in israeli dairy herds. Isr. J. Vet. Med. 70:16-21.

Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Vet. J. 176:50-57.

Goff, J. P. 2014. Calcium and magnesium disorders. Vet. Clin. North Am. Food Anim. Pract. 30:359-381.

Grohn, Y. T., S. W. Eicker, V. Ducrocq, and J. A. Hertl. 1998. Effect of diseases on the culling of Holstein dairy cows in New York State. J. Dairy Sci. 81:966-978.

Gröhn, Y. T., D. J. Wilson, R. N. Gonzalez, J. A. Hertl, H. Schulte, G. Bennett, and Y. H. Schukken. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 87:3358-3374.

Harmon, R. J. 1994. Physiology of mastitis and factors affecting somatic cell counts. J. Dairy Sci. 77:2103-2112.

Additional unpublished work

Jawor, P. E., J. M. Huzzey, S. J. LeBlanc, and M. A. von Keyserlingk. 2012. Associations of subclinical hypocalcemia at calving with milk yield, and feeding, drinking, and standing behaviors around parturition in Holstein cows. J. Dairy Sci. 95:1240-1248.

Kimura, K., T. A. Reinhardt, and J. P. Goff. 2006. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. J. Dairy Sci. 89:2588-2595.

Martin-Tereso, J. and H. Martens. 2014. Calcium and magnesium physiology and nutrition in relation to the prevention of milk fever and tetany (dietary management of macrominerals in preventing disease). Vet. Clin. North Am. Food Anim. Pract. 30:643-670.

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvao, and J. E. Santos. 2012. Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. J. Dairy Sci. 95:7158-7172.

Nordlund, K. V. 2006. Transition Cow Index. Pages 139 - 143 in 39th Proc. Am. Assoc. Bovine Practitioners. St. Paul, MN. Frontier Printers, Stillwater, OK.

Oetzel, G. R. and B. E. Miller. 2012. Effect of oral calcium bolus supplementation on early-lactation health and milk yield in commercial dairy herds. J. Dairy Sci. 95:7051-7065.

Pinedo, P., J. Velez, G. Solano, N. Rodriguez, J. Naves, G. M. Schuenemann, and C. Risco. 2017. Effect of oral calcium administration on the cure and reproductive performance of Holstein cows diagnosed with puerperal metritis. J. Dairy Sci. 100:2917-2927.

Rajala-Schultz, P. J., Y. T. Grohn, and C. E. McCulloch. 1999. Effects of milk fever, ketosis, and lameness on milk yield in dairy cows. J. Dairy Sci. 82:288-294.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Vet. J. 188:122-124.

Ribeiro, E. S., F. S. Lima, L. F. Greco, R. S. Bisinotto, A. P. Monteiro, M. Favoreto, H. Ayres, R. S. Marsola, N. Martinez, W. W. Thatcher, and J. E. Santos. 2013. Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. J. Dairy Sci. 96:5682-5697.

Roberts, T., N. Chapinal, S. J. Leblanc, D. F. Kelton, J. Dubuc, and T. F. Duffield. 2012. Metabolic parameters in transition cows as indicators for early-lactation culling risk. J. Dairy Sci. 95:3057-3063.

Additional unpublished work

Seifi, H. A., S. J. Leblanc, K. E. Leslie, and T. F. Duffield. 2011. Metabolic predictors of post-partum disease and culling risk in dairy cattle. Vet. J. 188:216-220.

Tenhagen, B. A., A. Helmbold, and W. Heuwieser. 2007. Effect of various degrees of dystocia in dairy cattle on calf viability, milk production, fertility and culling. J. Vet. Med. 54:98-102.

Venjakob, P. L., S. Borchardt, and W. Heuwieser. 2017. Hypocalcemia-Cow-level prevalence and preventive strategies in German dairy herds. J. Dairy Sci. 100:9258-9266.

Wilhelm, A. L., M. G. Maquivar, S. Bas, T. A. Brick, W. P. Weiss, H. Bothe, J. S. Velez, and G. M. Schuenemann. 2017. Effect of serum calcium status at calving on survival, health, and performance of postpartum Holstein cows and calves under certified organic management. J. Dairy Sci. 100:3059-3067.

5   DISCUSSION

The overall objective of this thesis was (1) to evaluate ear skin temperature as a cow-side test to predict serum calcium concentration, (2) to determine the prevalence of clinical and subclinical hypocalcemia in German dairy herds, and (3) to evaluate the impact of hypocalcemia on early lactation milk yield, reproductive performance, and culling risk in early lactation.

The results from the first study demonstrated that there was only a moderate relationship between ear skin temperature and blood calcium concentration. While hypothermia was more pronounced in cows with milk fever, ear skin temperature cannot be used as a cow-side test to detect subclinical hypocalcemia. It has been reported that cold ears were indicative for a low serum calcium concentration in dairy cows (Guterbock, 2004; Radostits et al., 2007; Peek and Divers, 2008). Larsen et al. (2001) reported an association of rectal temperature and blood calcium concentration in periparturient cows. Analyzing the association between skin temperature between the hip crests and blood calcium concentration however, a significant effect could not be demonstrated. Ambient temperature was a major confounder in my study. This observation is supported by a study of Gloster et al. (2011) in which the temperature of hooves in horses and claws in cows was evaluated, reporting that temperature of the extremities depended considerably on ambient temperature.

Currently, there is no cow-side test available to measure serum calcium concentration. Due to the short risk period of 12 to 24 h after parturition (Kimura et al., 2006), it has been recommended to give calcium orally twice, i.e., once at calving and 12 to 24 h later (Oetzel, 2013). This strategy was shown to be economically beneficial (McArt and Oetzel, 2015) when applied to all multiparous cows or cows with known risk factors (i.e., high milk yield in the previous lactation and/or lame). A selective treatment approach based on blood calcium concentration using a cow-side test might be even more beneficial. Due to the insufficient association between ear skin temperature and serum calcium concentration, further research needs to be conducted to provide a valid cow-side test. Assessment of the ear skin temperature should not be used as an indicator to apply calcium. In the meantime, individual cows can only be treated based on the lactation number and other risk factors for subclinical hypocalcemia.

The objective of the second study was to evaluate the prevalence of clinical and subclinical hypocalcemia considering modern genetics, husbandry and management conditions in Germany. Blood samples were drawn from fresh cows 0 to 48 h after parturition. In order to avoid bias by herd management, 12 blood samples were randomly selected from each participating farm (n = 115) and were enrolled in final statistical analyses (n = 1,380).

Discussion

Clinical milk fever and subclinical hypocalcemia were present in 8.6 % and 47.6 % of multiparous cows, respectively.

In contrast to multiparous cows, only a small proportion of primiparous cows (5.7 %) suffered from subclinical hypocalcemia in our study. A possible explanation for this might be that primiparous cows produce less colostrum and therefore have less requirement for calcium (Klingbeil, 2015). It is further speculated that a greater presence of active osteoblasts in the primiparous cow leads to a faster response to the release of PTH (Goff, 2014). Horst et al. (1990) indicate that also the number of receptors for 1,25-dihydroxyvitamin-D in the intestine is reduced in older cows.

The most cited study for the prevalence of subclinical and clinical hypocalcemia is from Reinhardt et al. (Reinhardt et al., 2011). Results from this study were based on blood samples taken in 2002 as part of the NAHMS dairy study. Our finding of 47.6 % of multiparous cows suffering from subclinical hypocalcemia is consistent with the observations from Reinhardt et al. (2011), who reported that 47 % of multiparous cows were suffering from subclinical hypocalcemia using a threshold of 2.0 mmol/L. Among first lactation cows however, Reinhardt et al. (2011) reported a proportion of 25 % being affected by subclinical hypocalcemia. In my study, considerably less primiparous cows were affected by subclinical hypocalcemia. The reason for this remains unclear. The NAHMS report from 2002 and 2014 stated, however, that feeding of anionic salts in heifers increased from 14.3 to 20.7 %, indicating that awareness for prevention of hypocalcemia has increased among farmers (USDA, 2002; 2014).

The study of Reinhardt et al. (2011) and my study are both based on convenience samples. Therefore, the results can hardly be compared as they might be biased by the selection of the farms participating. In my data set, 13.4 %, 15.0 %, and 21.7 % of fourth, fifth, and ≥ sixth parity cows suffered from milk fever, respectively. In comparison, in the study from Reinhardt et al. (2011), 10 %, 8 %, and 13 % of fourth, fifth, and sixth parity cows, respectively, were recumbent. Irrespective of parity, milk fever was prevalent in the study of Reinhardt et al. (2011) and mine in 5 % and 7.2 % of cows enrolled, respectively. In the US, subclinical hypocalcemia was present in 41 %, 49 %, 51 %, 54 %, and 42 % of second, third, fourth, fifth and ≥ sixth lactation cows, respectively. In comparison, prevalence of subclinical hypocalcemia was 29.0 %, 49.4 %, 60.4 %, 60.5 % and, 56.3 % in second, third, fourth, fifth and ≥ sixth lactation cows in my study, respectively.

While recumbent cows should be treated with calcium intravenously, the best way to treat cows with subclinical hypocalcemia is oral calcium supplementation (Oetzel, 2013; Blanc et al., 2014). There are different commercial oral calcium products available as calcium boluses or gels that can be easily supplemented to the fresh cow (Goff, 1999). Further evidence exists

that feeding of anionic salts in the close-up diet can reduce susceptibility to hypocalcemia (Leno et al., 2017a). In my study, oral calcium supplementation and feeding of anionic salts were implemented on 46.1 % and 8.7 % of the farms enrolled, respectively. In the US, 68.9 % and 27.6 % of the farms enrolled used calcium supplementation and feeding of anionic salts as preventive strategy, respectively (USDA, 2014).

The aim of the third study was to evaluate the impact of periparturient hypocalcemia on early lactation production and animal health. Analyzing the impact of subclinical hypocalcemia on milk production until 90 DIM, primiparous cows had a tendency to produce 2.07 kg/d less milk. In contrast, multiparous cows affected by subclinical hypocalcemia tended to produce 0.52 kg/d more milk. The question whether subclinical hypocalcemia affects milk production cannot be answered satisfyingly. While another study found a negative effect on milk production (Chapinal et al., 2012a), other authors reported no effect on milk yield (Martinez et al., 2012) and two studies even found evidence for higher milk yields in cows suffering from subclinical hypocalcemia (Jawor et al., 2012; Gild et al., 2015). None of these studies, however, considered milk yield in previous lactation as an important risk factor for hypocalcemia (Fleischer et al., 2001). By ignoring this, the association between milk yield and hypocalcemia might be biased. Nordlund et al. (2006) demonstrated that cows with high milk yield also produce milk yields above-average in the subsequent lactation. As milk yield of the previous lactation was considered in the model for multiparous cows in my study the results might not be biased. Evaluating the impact of milk fever on early lactation milk yield, there was a negative effect on early lactation milk yield in cows affected by milk fever. Cows that suffered from milk fever produced 1.9 kg/d less milk during the first 90 DIM.

Hypocalcemia 0 to 48 h postpartum had a negative impact on reproductive performance. Pregnancy per AI at first AI was decreased when cows were suffering from hypocalcemia (OR = 0.56; P = 0.001). This finding is consistent with a previous study in which serum calcium concentration of fresh cows was measured on the first three DIM. Cows were categorized into normocalcemic (all three values above 2.15 mmol/L), subclinical hypocalcemia (one or two of the values ≤ 2.15 mmol/L) and chronic subclinical hypocalcemia (all values ≤ 2.15 mmol/L) animals. It took longer for cows affected by chronic subclinical hypocalcemia to return to cyclic ovarian activity. Odds for becoming pregnant were decreased in these cows, as well.

In my data set, the hazard of getting pregnant within 150 DIM was also significantly reduced. On average, it took 15 d longer for hypocalcemic cows to get pregnant compared to normocalcemic cows. As it has been shown by Caixeta et al. (2015), there is further evidence that estrus cyclicity is delayed in cows affected by hypocalcemia (Ribeiro et al., 2013), which might also explain delayed pregnancy.

Discussion

Ultimately, the third study demonstrated that cows with subclinical and clinical hypocalcemia postpartum had 1.6 and 1.9 greater hazards of getting culled until 60 DIM, respectively. Recent literature has shown that periparturient hypocalcemia was associated with a number of secondary problems. Hypocalcemia is known to reduce muscle tone leading to recumbency and ultimately death, when serum calcium concentration decreases below 1.4 mmol/L and the animal is not treated efficiently (DeGaris and Lean, 2008). Also, in animals with subclinical hypocalcemia, rumen and abomasel motility as well as feed intake was reduced (Goff, 2008), leading to an increased risk for displaced abomasum (Chapinal et al., 2011; Seifi et al., 2011; Rodriguez et al., 2017). Immune function was impaired as peripheral mononuclear cells do not function properly in a state of low serum calcium concentration (Kimura et al., 2006; Martinez et al., 2012), causing higher susceptability to retained placenta (Rodriguez et al., 2017; Wilhelm et al., 2017), metritis (Martinez et al., 2012; Ribeiro et al., 2013; Rodriguez et al., 2017; Wilhelm et al., 2017), and mastitis (Curtis et al., 1983). Unsurprisingly, these negative implications lead to a higher risk for culling in early lactation. Our results are in agreement with Roberts et al. (2012), who proved that serum calcium concentration ≤ 2.2 and 2.3 mmol/L one and two weeks postpartum, respectively, was associated with 1.5 and 2.3 greater odds of getting culled in the first 60 DIM. A further study found even greater odds (2.4 and 5.3 when serum calcium concentration was ≤ 2.2 one week postpartum and ≤ 2.3 mmol/L two weeks postpartum, respectively) of getting culled in early lactation when blood calcium postpartum was below the threshold (Seifi et al., 2011). As stated previously such long risk periods, however, should be interpreted with caution.

Overall, the results of the three studies contribute to a better understanding of this important metabolic disorder that is linked to a variety of secondary problems. Prevalence of clinical and subclinical hypocalcemia in German dairy farms was high. My thesis clearly shows that periparturient hypocalcemia had a great impact on early lactation health, production and reproductive performance. The results presented herein stress that further research needs to be conducted in order to develop a validated cow-side test to raise awareness of this important issue and to give veterinarians and farmers the opportunity to apply a direct and selective treatment approach. Additionally, preventive strategies should be implemented more widely on German dairy farms.

6   SUMMARY

Diagnosis and prevalence of periparturient hypocalcemia and associated effects on milk production, reproductive performance and health of dairy cows in early lactation

The overall objectives of this thesis were (1) to evaluate ear skin temperature as a cow-side test to predict serum calcium concentration, (2) to determine the prevalence of clinical and subclinical hypocalcemia in German dairy herds, and (3) to evaluate the impact of hypocalcemia on early lactation milk yield, reproductive performance, and culling risk in early lactation.

To evaluate ear skin temperature as a cow-side test, a cross-sectional study was conducted enrolling 251 cows from 7 dairy herds. Skin temperature was scored manually by palpation of the ears and measured by infrared thermography. In addition, skin temperature on the coxal tubers, ambient temperature and rectal temperature were measured. Finally, a blood sample was drawn to determine serum calcium concentration. Cows with a serum calcium concentration below 2.0 mmol/L were considered as hypocalcemic. When clinical symptoms of hypocalcemia (e.g. shivering, recumbency) were apparent, the cow was classified as suffering from milk fever. Hypocalcemia in the absence of clinical symptoms was classified as subclinical hypocalcemia. To evaluate whether serum calcium concentration can be predicted by measuring the ear temperature and other temperature estimates, a multivariate analysis using the GENLINMIXED procedure and a ROC-curve analysis was performed. Hypocalcemia was prevalent in 3.3 %, 27.3 %, 32.8 % and 69.6 % of first, second, third and ≥ fourth lactation cows, respectively. In third and ≥ flourth lactation cows, 6.0 % and 20.3 % were suffering from clinical milk fever. Calcium status affected ear skin temperature. Normocalcemic cows (median 30,3 °C, IQR 26,0 °C – 32,8 °C) had higher ear skin temperature compared with cows affected by subclinical hypocalcemia (median 27.6 °C, IQR 22.1 °C – 30.8 °C) and milk fever (median 21.8 °C; IQR 14.7 °C – 27.0 °C). A decrease of 0.1 mmol/L in serum calcium concentration was associated with a decrease in ear temperature of 0.39 °C (95 % CI 0.25 – 0.54; *P* = 0.001). Ear skin temperature is not useful as a diagnostic test (sensitivity = 49.3; specificity = 73.8; threshold 2.0 mmol/L) to identify cows with subclinical hypocalcemia because of the great impact of ambient temperature on temperature of the ears. When there was an increase in ambient temperature of 1 °C, temperature of the ears rose by 0.78 °C (95 % CI 0.67 – 0.90; *P* = 0.001). Although there has been a mild association between serum calcium concentration and ear skin temperature, measurement of ear skin temperature cannot be recommended as a cow-side test to identify cows with subclinical hypocalcemia.

The objective of the second study was to evaluate prevalence of clinical and subclinical hypocalcemia in German dairy herds. In a cross-sectional study, blood samples were drawn

Summary

from periparturient cows 0 to 48 h after calving and analyzed for serum calcium, phosphorus and magnesium concentration. Overall, 1,709 blood samples were taken from 125 farms. Of these 329 blood samples had to be excluded, resulting in 1,380 blood samples from 115 farms (12 animals were tested on each farm). Cows were considered as normocalcemic or hypocalcemic using 2.0 mmol/L as a threshold. Cows with clinical signs (e.g. recumbency) were classified as milk fever. Each of the 115 herds was classified into negative (0 to 2/12), borderline (3 to 5/12), and positive ($\geq$ 6/12) according to the number of animals with hypocalcemia. Preventive strategies against hypocalcemia implemented on the farms were documented. Prevalence of clinical milk fever was 1.4 %, 5.7 % and 16.1 % for second, third, and $\geq$ fourth parity cows, respectively. None of the cows in first lactation was suffering from clinical milk fever. Subclinical hypocalcemia was present in 5.7 %, 29.0 %, 49.4 % and 60.4 % of cows in first, second, third, and $\geq$ fourth lactation, respectively. Fourteen, 51, and 50 herds were classified as negative, borderline, and positive, respectively. A positive correlation was observed between serum calcium and serum phosphorus concentration (R^2 = 0.335; *P* < 0.001). Serum calcium and serum magnesium concentration showed a negative correlation (R^2 = 0.151; *P* < 0.001). Of the farms enrolled, 65 had no preventive strategy implemented to control hypocalcemia. Among the farms with an implemented control strategy (n = 50), oral calcium supplementation at parturition was most common (40/50), followed by feeding of anionic salts in the close-up diet (10/50). The results indicate that prevalence of clinical and subclinical hypocalcemia in German dairy herds was high and that the minority of the farms had preventive strategies implemented.

The objective of the third study was to evaluate the impact of hypocalcemia on milk yield, reproductive performance, and culling. Herdsmen of the farms that participated in the previous study were asked to provide a backup of their herd management software 150 days after the last cow was sampled. After discarding cows (n = 283) with missing data, a total of 1,426 cows were considered for final analyses. Serum calcium concentration of each cow was related to early lactation milk yield (test day 1 to 3), reproductive performance (DIM at first artificial insemination (AI), pregnancy per AI at first AI, time to pregnancy within 150 DIM) and culling (until 60 DIM) data. Generalized linear mixed models were used to analyze continuous or categorical data. Shared frailty models were used for time to event data. Five different thresholds were used to define hypocalcemia. Thresholds ranged from 1.8 to 2.2 mmol/L using 0.1 mmol/L increments. Milk fever was defined as in the previous studies. Regarding the effect of hypocalcemia on milk yield, the effect was conditional on parity. While primiparous cows suffering from hypocalcemia (threshold: 2.0 mmol/L) tended to produce less milk (2.07 kg/d, *P* = 0.063) compared with normocalcemic primiparous cows in early lactation, hypocalcemic multiparous cows (threshold 2.1 mmol/L) had a tendency to produce more milk (0.51 kg/d, *P* = 0.086) compared with normocalcemic multiparous cows. Multiparous cows with clinical

hypocalcemia produced 1.90 kg/d (P = 0.002) less milk compared with normocalcemic cows in early lactation. Hypocalcemia did not affect time to first insemination (P = 0.296). Cows suffering from hypocalcemia (threshold 1.9 mmol/L) had decreased odds (OR 0.56; P = 0.001) of pregnancy at first artificial insemination. Calcium status had a significant impact on time to pregnancy (threshold 1.8 mmol/L). Median time to pregnancy was 109 and 134 days for normocalcemic and hypocalcemic animals, respectively. Cows with hypocalcemia (threshold: 2.0 mmol/L) and cows suffering from milk fever had a 1.6 (P = 0.019) and 1.9 (P = 0.029) times greater hazard of being culled within the first 60 DIM compared with normocalcemic animals. Giving the high prevalence of hypocalcemia and the associated negative outcomes, this study re-emphasizes the need to establish control strategies for hypocalcemia.

Overall, this thesis shows that there is high prevalence of hypocalcemia on German dairy farms. The results underline that periparturient hypocalciemia has a great impact on early lactation health, reproduction and production. Since measurement of ear skin temperature cannot be used as a diagnostic test, it has to be emphasized that there is an urgent need to develop a reliable cow-side blood calcium test.

7   ZUSAMMENFASSUNG

Diagnostik und Prävalenz geburtsnaher Hypokalzämie und deren Auswirkung auf die Milchleistung, Fruchtbarkeit und Gesundheit in der Frühlaktation von Milchkühen

Ziel dieser Untersuchung war es, (1) zu beurteilen, ob durch Messung der Ohrtemperatur ein Rückschluss auf die Konzentration von Kalzium im Blutserum der Kuh gezogen werden kann, (2) die Prävalenz von Milchfieber und subklinischer Hypokalzämie in deutschen Milchviehherden zu ermitteln und (3) den Einfluss von Hypokalzämie auf die Milchleistung (bis zum 90. Laktationstag), die Fruchtbarkeit und das Risiko für eine Milchkuh bis zum 60. Tag der Laktation gemerzt zu werden, zu beurteilen.

Zur Untersuchung, ob die Ohrtemperatur zur Identifikation hypokalzämischer Tiere verwendet werden kann, wurde eine Querschnittsstudie durchgeführt. 251 Tiere von sieben kommerziellen Milchviehbetrieben wurden in die Untersuchung einbezogen. Die Temperatur der Ohren wurde zunächst manuell mittels Palpation beurteilt und anschließend mit einem Infrarotthermometer gemessen. Zusätzlich wurde die Temperatur auf den Hüfthöckern, die Umgebungstemperatur und die Rektaltemperatur gemessen. Kühe mit einer Serumkonzentration von Kalzium von unter 2,0 mmol/L wurden als hypokalzämisch klassifiziert. Wenn klinische Anzeichen eines Kalziummangels vorhanden waren (z.B. Zittern, Festliegen), wurde dies als klinisches Milchfieber angesehen. Um zu beurteilen, ob ein Kalziummangel an Hand der Ohrtemperatur erkennbar ist, wurden Gemischte Lineare Modelle und eine ROC-Kurven Analyse durchgeführt. Mit steigender Laktationsnummer häufte sich das Auftreten von Kalziummangel. Es waren 3,3 % der Erstkalbinnen betroffen. Unter den Mehrkalbskühen hatten 27,3 % der Kühe in der zweiten Laktation, 32,8 % der Kühe in der dritten Laktation und 69,6 % der Tiere ab der vierten Laktation einen Kalziummangel. Milchfieber trat auf bei 6,0 % der Kühe in der dritten Laktation und bei 20,3 % der Tiere ab der vierten Laktation. Der Kalziumstatus hatte einen Einfluss auf die Ohrtemperatur. Normokalzämische Kühe (Median 30,3 °C, IQR 26,0 °C – 32,8 °C) hatten eine höhere Ohrtemperatur im Vergleich zu Kühen mit einer subklinischen Hypokalzämie (Median 27,6 °C; IQR 22,1°C – 30,8 °C) und Milchfieber (Median 21,8 °C; IQR 14,7 °C – 27,0 °C). Eine Absenkung der Serumkonzentration von Kalzium um 0,1 mmol/L war verbunden mit einer niedrigeren Ohrtemperatur von 0,39 °C (95 % CI 0,25 – 0,54; *P* = 0,001). Die Ohrtemperatur eignete sich nicht als diagnostischer Test (Sensitivität 49,3; Spezifität 73,8; Grenzwert 2,0 mmol/L) für subklinische Hypokalzämie, da die Umgebungstemperatur einen zu großen Einfluss auf die Ohrtemperatur hatte. Wenn die Außentemperatur um ein Grad anstieg, stieg auch die Ohrtemperatur um 0,78 Grad an (95 CI 0,67 – 0,90; *P* = 0,001).

Zusammenfassung

Obwohl es einen moderaten Zusammenhang zwischen Ohrtemperatur und der Serumkonzentration von Kalzium im Blut gab, kann die Ohrtemperatur nicht empfohlen werden, um Kühe mit subklinischer Hypokalzämie im geburtsnahen Zeitraum zu identifizieren.

Das Ziel der zweiten Studie war es, die Prävalenz für Milchfieber und subklinische Hypokalzämie zu ermitteln. In einer weiteren Querschnittsstudie wurden Blutproben von Kühen, 0 bis 48 Stunden nach der Kalbung auf Ihre Serumkonzentration von Kalzium, Phosphat und Magnesium im Blut untersucht. Insgesamt wurden von 1709 Tieren von 125 Betrieben Blutproben gezogen. Von diesen wurden 329 von der Studie ausgeschlossen und somit 1380 Blutproben von 115 Betrieben berücksichtigt (12 Kühe pro Betrieb). Kühe wurden als normokalzämisch betrachtet, wenn ihre Serumkonzentration von Kalzium im Blut ≥ 2,0 mmol/L war. Traten klinische Anzeichen von Hypokalzämie auf, so wurde dies als Milchfieber betrachtet. Jeder Betrieb wurde anhand des Auftretens von Kalziummangel in der Stichprobe als negativ (0 bis 2 Tiere hypokalzämisch), grenzwertig (3 bis 5 Tiere hypokalzämisch) oder positiv (6 bis 12 Tiere hypokalzämisch) für Hypokalzämie eingeordnet. Es wurde dokumentiert, ob der Betrieb systematische Prophylaxemaßnahmen gegen Hypokalzämie ergreift. Milchfieber trat bei 1,4 % der Zweitlaktierenden, bei 5,7 % der Tiere in der dritten Laktation und bei 16,1 % der Tiere ab der vierten Laktation auf. Mit steigender Laktationsnummer stieg auch das Risiko für subklinische Hypokalzämie. In der ersten Laktation lag die Prävalenz von subklinischer Hypokalzämie bei 5,7 %, in der zweiten bei 29,0 %, in der dritten bei 49,4 % und ab der vierten bei 60,4 %. Vierzehn Herden wurden anhand des Auftretens von Kalziummangel in der Stichprobe als negativ für Hypokalzämie, 51 als grenzwertig und 50 als positiv eingeordnet. Die Serumkonzentration von Kalzium und Phosphat waren positiv miteinander korreliert (R^2 = 0,335; *P* < 0,001). Dagegen gab es eine negative Korrelation zwischen Kalzium und Magnesium (R^2 = 0,151; *P* < 0,001). Unter den teilnehmenden Betrieben führten 65 Prophylaxemaßnahmen gegen geburtsnahen Kalziummangel durch. Die etablierten Phrophylaxemaßnahmen (n = 50) waren vorwiegend die orale Applikation von Kalzium (40/50), gefolgt von der Fütterung von sauren Salzen an die Vorbereitergruppe (10/50).

Die Ergebnisse zeigen, dass die Prävalenz von subklinischer Hypokalzämie und Milchfieber auf deutschen Milchviehbetrieben hoch war und dass weniger als die Hälfte der Betriebe Maßnahmen ergriffen, um einen geburtsnahen Kalziummangel zu vermeiden.

Das Ziel der dritten Studie war es, den Einfluss von Hypokalzämie auf die Milchleistung (bis zu 90. Laktationstag), Fruchtbarkeit und das Risiko für eine Milchkuh bis zum 60. Laktationstag gemerzt zu werden, zu beurteilen. Die Betriebsleiter der Betriebe, die an der vorangehenden Studie teilgenommen haben, wurden 150 Tage nachdem die letzte Kuh getestet wurde, kontaktiert und gebeten eine Datensicherung der Betriebssoftware zur Verfügung zu stellen. Nachdem 283 Tiere auf Grund von fehlenden Daten ausgeschlossen

wurden, konnten die Daten von 1426 Kühen ausgewertet werden. Die Serumkonzentration von Kalzium wurde mit Milchleistungs- (bis zur dritten Milchleistungsprüfung, (MLP)), Fruchtbarkeits- (Rastzeit, Erstbesamungserfolg, Güstzeit) und Merzungsdaten (bis zum 60. Laktationstag) in Beziehung gesetzt. Mit Hilfe von Gemischten Linearen Modellen wurden kontinuierliche und kategoriale Daten ausgewertet. Für die anderen Parameter (Rastzeit, Güstzeit, Merzung innerhalb von 60 Laktationstagen) wurden Überlebenszeitanalysen (Shared Frailty Modelle) verwendet. Die verwendeten Grenzwerte für Kalzium zur Definition von subklinischer Hypokalzämie reichten von 1,8 bis 2,2 mmol/L. Milchfieber wurde wie in den vorangehenden Studien definiert. Der Effekt von Hypokalzämie auf die Milchleistung unterschied sich zwischen Erstkalbinnen und Mehrkalbskühen. Erstkalbinnen mit Hypokalzämie (Grenzwert 2,0 mmol/L) hatten tendenziell weniger Milch (-2,07 kg/d bis zur dritten MLP; P = 0,063) als normokalzämische Tiere in der ersten Laktation. Hypokalzämische Mehrkalbskühe (Grenzwert 2,1 mmol/L) produzierten dagegen tendenziell mehr Milch (0,51 kg/d bis zur dritten MLP; P = 0,086) als normokalzämische Mehrkalbskühe. Tiere mit klinischem Milchfieber produzierten bis zur dritten MLP 1,90 kg/d (P = 0,001) weniger Milch als Tiere, die keinen Kalziummangel zur Geburt aufwiesen. Die Rastzeit wurde nicht durch das Vorhandensein von Hypokalzämie beeinflusst (P = 0,296). Dagegen hatten Kühe mit Hypokalzämie (Grenzwert 1,9 mmol/L) verminderte Chancen (Odds Ratio (OR) = 0,56; P = 0,001) bei der ersten Besamung tragend zu werden. Darüber hinaus hatte der Kalziumstatus einen signifikanten Einfluss auf die Güstzeit (Grenzwert = 1,8 mmol/L). Bei normokalzämischen Tieren lag der Median der Güstzeit bei 109 Tagen, bei hypokalzämischen dagegen bei 134 Tagen. Kühe mit subklinischer Hypokalzämie (Grenzwert 2,0 mmol/L) hatten eine 1,6-fach erhöhte Gefahr in den ersten 60 Laktationstagen gemerzt zu werden. Bei Kühen mit Milchfieber war die Gefahr gemerzt zu werden 1,9-fach erhöht.

Die durchgeführten Studien zeigen, dass die Prävalenz von Hypokalzämie auf deutschen Milchviehbetrieben hoch ist. Die Ergebnisse unterstreichen, dass Kalziummangel im geburtsnahen Zeitraum einen negativen Effekt auf Milchproduktion, Fruchtbarkeit und die Gesundheit in der Frühlaktation hat. Da die Messung der Ohrtemperatur als Schnelltest für Hypokalzämie unbrauchbar ist, ist es dringend erforderlich einen zuverlässigen Schnelltest zu entwickeln, der im Stall angewendet werden kann.

8   REFERENCES FOR INTRODUCTION AND DISCUSSION

Blanc, C. D., M. Van der List, S. S. Aly, H. A. Rossow, and N. Silva-del-Rio. 2014. Blood calcium dynamics after prophylactic treatment of subclinical hypocalcemia with oral or intravenous calcium. J. Dairy Sci. 97:6901-6906.

Caixeta, L. S., P. A. Ospina, M. B. Capel, and D. V. Nydam. 2015. The association of subclinical hypocalcemia, negative energy balance and disease with bodyweight change during the first 30 days post-partum in dairy cows milked with automatic milking systems. Vet. J. 204:150-156.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. J. Dairy Sci. 94:4897-4903.

Chapinal, N., M. E. Carson, S. J. LeBlanc, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012a. The association of serum metabolites in the transition period with milk production and early-lactation reproductive performance. J. Dairy Sci. 95:1301-1309.

Chapinal, N., S. J. Leblanc, M. E. Carson, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012b. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. J. Dairy Sci. 95:5676-5682.

Curtis, C. R., H. N. Erb, C. J. Sniffen, R. D. Smith, P. A. Powers, M. C. Smith, M. E. White, R. B. Hillman, and E. J. Pearson. 1983. Association of parturient hypocalcemia with eight periparturient disorders in Holstein cows. J. Am. Vet. Med. Assoc. 183:559-561.

DeGaris, P. J. and I. J. Lean. 2008. Milk fever in dairy cows: a review of pathophysiology and control principles. Vet. J. 176:58-69.

Fleischer, P., M. Metzner, M. Beyerbach, M. Hoedemaker, and W. Klee. 2001. The relationship between milk yield and the incidence of some diseases in dairy cows. J. Dairy Sci. 84:2025-2035.

Gild, C., N. Alpert, and M. van Straten. 2015. The Influence of Subclinical Hypocalcemia on Production and Reproduction Parameters in Israeli Dairy Herds. Isr. J. of Vet. Med. 70:16-21.

Gloster, J., K. Ebert, S. Gubbins, J. Bashiruddin, and D. J. Paton. 2011. Normal variation in thermal radiated temperature in cattle: implications for foot-and-mouth disease detection. BMC Vet. Res. 7:73.

References for Introduction and Discussion

Goff, J. P. 1999. Treatment of Calcium, Phosphorus, and Magnesium Balance Disorders. Vet. Clin. of North Am. Food Anim. Pract. 15:619-639.

Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Vet. J. 176:50-57.

Goff, J. P. 2014. Calcium and magnesium disorders. Vet. Clin. North Am. Food Anim. Pract. 30:359-381.

Guterbock, W. M. 2004. Diagnosis and treatment programs for fresh cows. Vet. Clin. North Am. Food Anim. Pract. 20:605-626, vii.

Horst, R. L., J. P. Goff, and T. A. Reinhardt. 1990. Advancing Age Results in Reduction of Intestinal and Bone 1,25-Dihydroxyvitamin D Receptor. Endocrinology 126:1153-1157.

Jawor, P. E., J. M. Huzzey, S. J. LeBlanc, and M. A. von Keyserlingk. 2012. Associations of subclinical hypocalcemia at calving with milk yield, and feeding, drinking, and standing behaviors around parturition in Holstein cows. J. Dairy Sci. 95:1240-1248.

Kimura, K., T. A. Reinhardt, and J. P. Goff. 2006. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. J. Dairy Sci. 89:2588-2595.

Klingbeil, M. 2015. Investigation of influence factors on yield, quality and calcium content of first colostrum in Holstein. Ruminant and Swine Clinic, Faculty of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany.

Larsen, T., G. Moller, and R. Bellio. 2001. Evaluation of clinical and clinical chemical parameters in periparturient cows. J. Dairy Sci. 84:1749-1758.

Leno, B. M., S. E. LaCount, C. M. Ryan, D. Briggs, M. Crombie, and T. R. Overton. 2017a. The effect of source of supplemental dietary calcium and magnesium in the peripartum period, and level of dietary magnesium postpartum, on mineral status, performance, and energy metabolites in multiparous Holstein cows. J Dairy Sci 100:7183-7197.

Leno, B. M., C. M. Ryan, T. Stokol, D. Kirk, K. P. Zanzalari, J. D. Chapman, and T. R. Overton. 2017b. Effects of prepartum dietary cation-anion difference on aspects of peripartum mineral and energy metabolism and performance of multiparous Holstein cows. J. Dairy Sci. 100:4604-4622.

Martin-Tereso, J. and H. Martens. 2014. Calcium and magnesium physiology and nutrition in relation to the prevention of milk fever and tetany (dietary management of macrominerals in preventing disease). Vet. Clin. North Am. Food Anim. Pract. 30:643-670.

References for Introduction and Discussion

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvao, and J. E. Santos. 2012. Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. J. Dairy Sci. 95:7158-7172.

Martinez, N., L. D. Sinedino, R. S. Bisinotto, E. S. Ribeiro, G. C. Gomes, F. S. Lima, L. F. Greco, C. A. Risco, K. N. Galvao, D. Taylor-Rodriguez, J. P. Driver, W. W. Thatcher, and J. E. Santos. 2014. Effect of induced subclinical hypocalcemia on physiological responses and neutrophil function in dairy cows. J. Dairy Sci. 97:874-887.

McArt, J. A. and G. R. Oetzel. 2015. A stochastic estimate of the economic impact of oral calcium supplementation in postparturient dairy cows. J. Dairy Sci. 98:7408-7418.

Nordlund, K. V. 2006. Transition Cow Index. Pages 139 - 143 in 39th Proc. Am. Assoc. Bovine Practitioners. St. Paul, MN. Frontier Printers, Stillwater, OK.

Oetzel, G. R. 2013. Oral calcium supplementation in peripartum dairy cows. Vet. Clin. North Am. Food Anim. Pract. 29:447-455.

Oetzel, G. R. and B. E. Miller. 2012. Effect of oral calcium bolus supplementation on early-lactation health and milk yield in commercial dairy herds. J. Dairy Sci. 95:7051-7065.

Peek, S. and T. Divers. 2008. Metabolic diseases. Pages 590-605 in Rebhuns Diseases in Dairy Cattle. ed. Saunders Elsevier, St. Louis, MO.

Pinedo, P., J. Velez, G. Solano, N. Rodriguez, J. Naves, G. M. Schuenemann, and C. Risco. 2017. Effect of oral calcium administration on the cure and reproductive performance of Holstein cows diagnosed with puerperal metritis. J. Dairy Sci. 100:2917-2927.

Radostits, O. M., H. K.W., and C. P.D. 2007. Veterinary Medicine. A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats. 10th Edition ed. W.B. Saunders Company Ltd, London, UK.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Vet. J. 188:122-124.

Ribeiro, E. S., F. S. Lima, L. F. Greco, R. S. Bisinotto, A. P. Monteiro, M. Favoreto, H. Ayres, R. S. Marsola, N. Martinez, W. W. Thatcher, and J. E. Santos. 2013. Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. J. Dairy Sci. 96:5682-5697.

References for Introduction and Discussion

Roberts, T., N. Chapinal, S. J. Leblanc, D. F. Kelton, J. Dubuc, and T. F. Duffield. 2012. Metabolic parameters in transition cows as indicators for early-lactation culling risk. J. Dairy Sci. 95:3057-3063.

Rodriguez, E. M., A. Aris, and A. Bach. 2017. Associations between subclinical hypocalcemia and postparturient diseases in dairy cows. J. Dairy Sci. 100:7427-7434.

Seifi, H. A., S. J. Leblanc, K. E. Leslie, and T. F. Duffield. 2011. Metabolic predictors of post-partum disease and culling risk in dairy cattle. Vet. J. 188:216-220.

USDA. 2002. Part I: Reference of Dairy Cattle Health and Health Management Practises in the Uninted States. APHIS, Fort Collins, CO.

USDA. 2014. Dairy Cattle Management Practices in the United States. APHIS, Fort Collins, CO.

Wilhelm, A. L., M. G. Maquivar, S. Bas, T. A. Brick, W. P. Weiss, H. Bothe, J. S. Velez, and G. M. Schuenemann. 2017. Effect of serum calcium status at calving on survival, health, and performance of postpartum Holstein cows and calves under certified organic management. J. Dairy Sci. 100:3059-3067.

9 PUBLICATIONS

Research articles

Venjakob, P. L.; S. Borchardt; G. Thiele and W. Heuwieser. (2016):
Evaluation of ear skin temperature as a cow-side test to predict postpartum calcium status in dairy cows.
Journal of Dairy Science. 99:6542–6549.

Venjakob, P. L.; G. Thiele; P.-H. Clausen and A. M. Nijhof (2017):
Toxocara vitulorum infection in German beef cattle.
Parasitology Research. 116:1085–1088.

Venjakob, P.L.; S. Borchardt and W. Heuwieser (2017):
Orale Kalziumgabe zur Hypokalzämie-Prophylaxe im geburtsnahen Zeitraum.
Der Praktische Tierarzt. 98:702–707

Venjakob P. L.; S. Borchardt and W. Heuwieser (2017):
Hypocalcemia—Cow-level prevalence and preventive strategies in German dairy herds.
Journal of Dairy Science. 100:9258–9266.

Venjakob P. L.; L. Pieper; W. Heuwieser and S. Borchardt (2018)
Association of postpartum hypocalcemia with early-lactation milk yield, reproductive performance, and culling in dairy cows.
Journal of Dairy Science. In press.

Oral presentations at conferences

Venjakob, P. (2016):
Möglichkeiten der postpartalen Hypocalcämie-Prophylaxe.
Update Fortpflanzung beim Rind, Tierklinik für Fortpflanzung, Freie Universität Berlin.
Berlin. 27.05.-28.05.2016.

Venjakob, P. L.; S. Borchardt and W. Heuwieser. (2016):
Evaluierung der Ohrtemperatur zur Diagnostik von subklinischer Hypocalcämie bei Milchkühen.

41. Leipziger Fortbildungsveranstaltung - Labordiagnostik in der Bestandsbetreuung.
Leipzig. 24.06.2016.

Borchardt, S.; P. L. Venjakob and W. Heuwieser. (2016):
Subklinische Hypocalcämie in deutschen Milchviehbetrieben - Häufigkeit und Auswirkung auf das Abgangsrisiko in der Frühlaktation.
bpt-Kongress 2016. In: bpt-Kongress 2016: Vortragsband – BPT Akademie GmbH (Hrsg.), p. 116–119
Hannover. 18.11.-19.11.2016.

Venjakob P. L.; G. Thiele; H.-P. Clausen and A. M. Nijhof. (2017):
Toxocara vitulorum in einer deutschen Mutterkuhherde.
DVG-Vet-Congress 2017
Berlin. 10.11. – 11.11.2017

Poster presentations at conferences

Venjakob, P. (2016):
Prevalence of subclinical hypocalcemia in German dairy herds.
29th World Buiatrics Congress. In: The 29th World Buiatrics Congress, Dublin 2016 - Congress Proceedings – Michael Doherty (Hrsg) S. 487
Dublin, Irland – 03.07.-08.07.2016.

Venjakob, P. L.; S. Borchardt and W. Heuwieser (2017):
Untersuchung zur Prävalenz von subklinischer Hypokalzämie in deutschen Milchviehherden.
DVG-Vet-Congress 2017
Berlin. 10.11. – 11.11.2017

10   ACKNOWLEDGEMENTS

11   DECLARATION OF INDEPENDENCE

Hiermit erkläre ich, dass ich, Peter Lennart Venjakob, alle Studien selbständig durchgeführt und die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Tabelle 1. Eigener Anteil[1] an den Forschungsprojekten der vorliegenden Dissertation

	Studie 1[a]	Studie 2[b]	Studie 3[c]
Studienplanung	+++	+++	+++
Datenerhebung	+++	+++	+++
Datenanalyse	+++	+++	+++
Verfassen des Manuskripts	+++	+++	+++
Editieren des Manuskripts	++	++	++

[1]Legende: +++: > 70 %

++:   50-70 %

+:   < 50 %

[a] Evaluation of ear skin temperature as a cow-side test to predict postpartum calcium status in dairy cows

[b] Hypocalcemia – Cow-level prevalence and preventive strategies in German dairy herds

[c] Impact of periparturient hypocalcemia on milk yield, reproductive performance and culling in dairy cows

Berlin, den 20. November 2017