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Evaluation of udder firmness and fecal cortisol metabolites as cow-side parameters in dairy cows

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Für meine Eltern und meinen Bruder

“Whoever needs milk, bows to the animal.”
(Yiddish Saying)

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1 INTRODUCTION

Animal welfare issues of food producing animals are of increasing significance both in research and to the general public (Rushen et al., 2007; von Keyserlingk et al., 2009, 2013; Barkema et al., 2015). As a result, there is a pressing need for the evaluation of practical, cow-based and standardized tools or parameters to objectively measure animal welfare and stress in dairy cows on-farm (Blokhuis et al., 2008; Tremetsberger and Winckler, 2015). The validity and reliability of these measures should be known (EFSA, 2012). Furthermore, the conversion of science-based welfare-related measures into information that is accessible for and easily understood by the consumer is needed (Blokhuis et al., 2008). A high level of standardization in parameter measurement also leads to a more reliable diagnosis of a specific condition or disease. Subsequently, a more specific and optimized treatment of a disease can be performed or a change of a condition can be followed up more accurately (Swinkels et al., 2015).

Clinical mastitis (CM) and heat stress are intensively investigated and highly relevant due to their importance for the dairy industry. Both have a negative effect on the welfare of dairy cows (Silanikove, 2000; Fitzpatrick et al., 2013). Furthermore, both cause substantial economic losses (St-Pierre et al., 2003; Heikkilä et al., 2012), result in behavioral changes such as lowered lying time (Allen et al., 2015; Fogsgaard et al., 2015) and negatively affect reproductive performance (Huang et al., 2008; Hertl et al., 2014).

Assessment of udder firmness is an essential part of a sound clinical examination of a dairy cow and a practical tool to detect CM promptly. However, data on repeatability or validity of methods to examine udder firmness in lactating cows have not been described except for one study (Houe et al., 2002). Therefore, validity of estimates of udder firmness in healthy cows was evaluated in a first study. A second study focused on udder firmness in cows suffering from CM.

Heat and humidity are well known to stress dairy cows (e.g., West, 2003). Measurement of fecal cortisol metabolites in fecal samples is a scientifically established and practical method to determine stress levels in animals (Palme et al., 1999; Morrow et al., 2002; Möstl and Palme, 2002). In dairy cows, fecal cortisol metabolites have already been used as an indicator for stress during the transition period (Huzzey et al., 2015), dry-off (Bertulat et al., 2013), handling (Saco et al., 2008) and transport (Palme et al., 2000). In this thesis, the focus is on a new field of application of measurement of fecal cortisol metabolites i.e., measurement of heat stress.

That said, the overall focus of this work was to evaluate two objective parameters [i.e., udder firmness and fecal glucocorticoid metabolites (11,17-dioxoandrostanes; 11,17-DOA)],

which can be assessed without stressful restraining and manipulating the cow, to verify two important issues in dairy cow management (i.e., CM and heat stress).

1.1 Evaluation of udder firmness by palpation and a dynamometer

Swelling and an increased firmness of the mammary gland are important signs of inflammation and associated with CM (IDF, 1999). Therefore, apart from visual observation of milk and implementation of a California Mastitis Test, manual palpation of the udder is a simple and quick method to detect CM within the daily milking routine (Hillerton, 2000; Pyörälä, 2003; IDF, 2007) and an important diagnostic criterion for cow-side treatment before laboratory results of milk samples are available (Lago et al., 2011). Palpation was used in numerous studies addressing CM in the dairy cow (e.g., Peters et al., 2015) to differentiate between cows without and with CM. Several of these studies use a palpation scoring system to assess udder firmness (Hogan et al., 1995; Gleeson et al., 2007; O'Driscoll et al., 2011; Petrovski et al., 2011; Scaletti and Harmon, 2012). Internationally recognized textbooks detail the method of palpation of the udder as an integral part of the clinical examination of an individual cow (Rosenberger et al., 1990) and the physical examination of the udder as a basic component of an udder health management program for dairy herds (Radostits et al., 2001). However, except for two studies estimating udder firmness with a technical device in dried-off (Bertulat et al., 2012) or by palpation in lactating cows (Houe et al., 2002), data on repeatability or validity of methods to examine udder firmness have not yet been described. Furthermore, palpation of the udder tissue as an essential part of cow-side mastitis diagnostics is not well defined in the current literature.

Therefore, the overall objective of the first study was to evaluate the validity of estimates of udder firmness determined by palpation and by using a dynamometer and to compare 4-point palpation scoring system with measures obtained with the dynamometer considering different factors (within-observer repeatability, between-observer repeatability, time of measurement, day of study).

Results of this study have been published in the Journal of Dairy Science (Impact Factor 2014: 2.573):

Rees, A., C. Fischer-Tenhagen, and W. Heuwieser. 2014. Evaluation of udder firmness by palpation and a dynamometer. Journal of Dairy Science 97:3488-3497.

1.2 Udder firmness and clinical mastitis

After evaluating udder firmness measurements in a first study, a follow-up trial was conducted to investigate if udder firmness can be used as a cow-side indicator for CM.

As indicated above, CM is a highly relevant disease in dairy cows (Hertl et al., 2011, 2014) and has been proven to be painful (Fitzpatrick et al., 2013). Furthermore, it is the most common indication for the use of antimicrobial agents in dairy cows (Thomson et al., 2008). Thus, this disease concerns top priority issues such as animal welfare (Barkema et al., 2015) and a prudent use of antibiotics (e.g., Oliver et al., 2011; Machado et al., 2014). Veterinarians and farmers frequently base treatment decisions on clinical symptoms of the udder (Swinkels et al., 2015). There is no science-based information, however, to quantitatively define a healthy udder using specific thresholds for udder firmness. Besides descriptions in textbooks (Rosenberger et al., 1990; Radostits et al., 2001), data are not available to objectively differentiate healthy from affected udders. Additionally, farmers' insecurity in mastitis therapy and wrong decisions regarding extended treatment of CM has been described most recently (Swinkels et al., 2015). Therefore, more research is warranted on the evolution of clinical criteria (Swinkels et al., 2015) and specific guidelines to provide differentiation between cows without and with CM are needed. The timely detection of signs of CM would also allow shorter and more effective drug treatments (Trevisi et al., 2014).

Therefore, the overall objective of this study was to evaluate if udder firmness can be used as a cow-side indicator for mastitis.

These data have not been published yet and are presented in section 3 "Additional unpublished work".

1.3 Heat stress and fecal cortisol metabolites

The second cow-side parameter evaluated in this thesis is 11,17-DOA as a possible indicator for heat stress measurable in feces.

An increasing milk yield per cow over the last decades (Hansen, 2000) has resulted in increased metabolic heat production (Kadzere et al., 2002). In particular high-yielding dairy cows became less tolerant to hot climate conditions (West, 2003). Furthermore, heat waves recently were proven to be associated with a higher risk of death in dairy cows (Vitali et al., 2015). Both the number of larger-scale dairy farms and herd size increased over the last decades (Winsten et al., 2010). Whereas the influence of heat stress on animal welfare of extensively managed cattle has been reviewed (Silanikove, 2000), there is a lack of information on the impact of heat stress on animal welfare of cows housed on these large dairy farms. A hormonal stress response in heat stressed cows i.e., elevated plasma cortisol concentrations measured via blood sampling, was already proven (Christison and Johnson, 1972; Elvinger et

al., 1992; Muller et al., 1994). Sampling feces instead of blood has the advantage of a stress-free handling of the cow (Möstl and Palme, 2002) and easy collection of samples. Concentrations of fecal glucocorticoid metabolites, blood cortisol, and adrenal activity are directly related (Palme et al., 1999; Morrow et al., 2002). These findings lead to the hypothesis that fecal cortisol can be used as an indicator for heat stress in cows.

Therefore, the overall objective of a third study was to evaluate if acute and chronic heat stress in individual dairy cows is associated with concentrations of fecal 11,17-DOA.

Results of this study have been published in the Journal of Reproduction in Domestic Animals (Impact Factor 2014: 1.515):

Rees, A., C. Fischer-Tenhagen, and W. Heuwieser. 2016. Effect of heat stress on concentrations of faecal cortisol metabolites in dairy cows. Reproduction in Domestic Animals 51:392-399.

2 PUBLICATION I

Evaluation of udder firmness by palpation and a dynamometer

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2.1 Abstract

Swelling of the mammary gland is an important health status sign for clinical exploration and palpation is a routine diagnostic tool for mastitis detection in dairy cows. Data on repeatability or validity of specific methods of udder palpation are rare. The overall objective was to study the validity of estimates of udder firmness generated by palpation and by using a validated dynamometer. Specifically, we set out to determine within-observer repeatability and between-observer repeatability in two specific experiments. Additionally, we compared a 4-point palpation scoring system with estimates obtained with a dynamometer in this study. In a pilot trial, we determined the range of udder firmness of 25 cows and developed an in-vitro model for udder firmness. This model enabled training of the observers and allowed investigating a 4-point palpation scoring system. In vivo, udder firmness was determined before and after milking by palpation and by using a dynamometer. Within-observer repeatability based on estimates of udder firmness of 25 cows obtained by three observers on a single day by palpation was 0.968. Within-observer repeatability of estimates of udder firmness of 25 cows obtained with the dynamometer by a single observer was 0.997. The coefficient of variation of the same measures was 9.1%. To determine between-observer repeatability (palpation: 0.932, dynamometer: 0.898), udder firmness of 100 cows was measured on four different days by nine observers in experiment 2. Udder firmness in dairy cows could be measured repeatably with the dynamometer and by palpation, especially when performed by a single observer. Estimates of udder firmness generated by palpation and with the dynamometer were moderately related (correlation coefficient: 0.54). Training of observers through the pilot trial or practical experience in the four days of the study in experiment 2 did not improve the correlation. Further research is warranted to understand how udder firmness develops in infected udders.

2.2 Key words

udder firmness, dynamometer, palpation, repeatability

2.3 Introduction

Swelling of the mammary gland is an important inflammation sign and is associated with clinical mastitis (IDF, 1999). Furthermore, udder swelling is a health status sign for clinical exploration. Clinical examination of the udder includes palpation of the udder tissue. Palpation is a routine diagnostic tool for mastitis detection in dairy cows (Hillerton, 2000; Pyörälä, 2003; IDF, 2007) and is a simple and quick method to diagnose relevant findings indicative of inflammation of the udder (i.e., nodes, heat, pain, and swelling). Palpation was used in

numerous studies addressing detection (Polat et al., 2010; Petrovski et al., 2011), prevention (Runciman et al., 2010), and antibiotic treatment (Cao et al., 2007; Lago et al., 2011) to differentiate between cows without and with mastitis. Apart from visual observation of milk and implementation of a California Mastitis Test, palpation is an important diagnostic criterion for cow-side treatment before cytological and bacteriological laboratory results of milk samples are available (Lago et al., 2011).

Internationally recognized textbooks detail the method of palpation of the udder as an integral part of the clinical examination of an individual cow (Rosenberger et al., 1990) and the physical examination of the udder as a basic component of an udder health management program for dairy herds (Radostits et al., 2001). Two 5-point palpation scoring systems have been developed to describe the severity of symptoms (Petrovski et al., 2011) or the clinical status of the quarters (Hogan et al., 1995; Scaletti and Harmon, 2012). A 3-point scoring system for udder firmness has been assessed to examine effects of changes of milking frequency (Gleeson et al., 2007) or omission of a milking event (O'Driscoll et al., 2011) on animal welfare. To achieve more objective measures of udder firmness, some studies used different technical devices (i.e., dynamometer) for measuring the force to indent the udder tissue for studying effects related to animal welfare (Tucker et al., 2007, 2009) and dry-cow management (Bertulat et al., 2013). Except for two studies estimating udder firmness with a technical device (Bertulat et al., 2012) or by palpation (Houe et al., 2002), data on repeatability or validity of methods to examine udder firmness have not been described. Furthermore, palpation of the udder tissue as an essential part of cow-side mastitis diagnostics is not well defined in the current literature.

Thus, the overall objective of our study was to evaluate the validity of estimates of udder firmness determined by palpation and by using a dynamometer. Specifically, we set out to 1) determine within-observer repeatability (WOR; experiment 1) and between-observer repeatability (BOR; experiment 2) of estimates of udder firmness generated by palpation and using a dynamometer, and 2) compare a 4-point palpation scoring system with measures obtained with a dynamometer.

2.4 Materials and methods

2.4.1 Animals

The study was conducted in September and October 2012 on a commercial dairy farm milking 175 dairy cows in Brandenburg, Germany. For the study, a total of 150 Holstein-Friesian and crossbreeds of Holstein-Friesian dairy cows (71 primiparous and 79 multiparous) were used. Cows were housed in a deep-bedded stall. They received a balanced TMR based

on 52.7% corn silage, 24.8% grass silage, 9.3% brewers grains, 5.3% corn meal, 4.7% rapeseed, 2.2% triticale, 0.9% straw, 0.1% urea, and basic mineral mix. The TMR was delivered twice per day at 0830 and 1700 h. Cows were milked twice daily (0700 and 1500 h) in a 2 x 8 Herringbone milking parlor (System Happel GmbH, Friesenried, Germany). The rolling herd average (305 days) was 8,745 ± 2,149 kg of milk/cow per year. The actual milk yield was 28.4 ± 8.4 kg/cow per day. Cows were in different stages of lactation (mean ± SD: 147 ± 94 DIM).

2.4.2 Measurements

Udder firmness was determined by palpation and by using a dynamometer (Penefel DFT 14; Agro Technologie, Forges-les-Eaux, France). The dynamometer was used following the standard operating procedure (SOP) described by Bertulat et al. (2012). In brief, the left hind udder quarter of cows enrolled was used to determine udder firmness. The measuring point was located in the horizontal and vertical center of the left hind quarter. This point was marked with livestock paint crayons (Raidex GmbH, Dettingen, Germany) to ensure a consistent measurement location within the udder. The cow had to stand with all four legs on a level surface during the whole measurement. After five consecutive measurements performed within 10 s, the dynamometer displayed the arithmetic mean and coefficient of variation. Values with a coefficient of variation exceeding 10% were discarded and the measurement repeated. This general procedure of measuring udder firmness with the dynamometer was identical in all experiments.

Palpation was conducted by pressing the fingertips of all fingers of one hand except the thumb into the udder tissue. The measuring point was identical to the marked measuring point for the dynamometer. This approach was selected to standardize the location within the udder and within the quarter to reduce bias due to inhomogeneity of the tissue.

2.4.3 Pilot trial

A pilot trial was conducted to determine the range of udder firmness expectable in healthy dairy cows considering different stages of lactation before and after milking. Furthermore, we wanted to investigate if observers were able to correctly classify different firmness levels of specimens presented in vitro on a 4-point palpation scoring system with sufficient repeatability before implementing a large field study.

In total, 25 cows were fixed in the head locker before and after the evening milking and udder firmness was determined using the dynamometer on 10 consecutive days. To avoid bias

due to recognizing an individual cow by the observer, cows were fixed in different positions after milking each day. All measurements were conducted within 45 min before and within 45 min after milking. Based on the range of udder firmness measured with the dynamometer, a 4-point classification system was developed by dividing the range determined by four and calculating the mean value of the estimates within that increment.

An in-vitro trial comprising a reference standard was conducted to enable the calculation of WOR and BOR for the 4-point classification system, which was also used in the in-vivo trial. As a reference standard, four tire tubes (30.48 cm; Schrader valve, TAQ-33 Technique + Quality; BICO Zweirad Marketing GmbH, Verl, Germany) were inflated. The firmness levels of the tires were adjusted such that measurements obtained with the dynamometer equaled the four means of udder firmness calculated for the 4-point classification system developed with data obtained in the pilot trial. As a result, each tire tube represented one distinct score of the 4-point system. To ensure repeatability of this experiment, air pressures measured with a manometer as reference standard (GDH 200-14; Greisinger Electronic GmbH, Regenstauf, Germany) were also reported (table 1). For example, tire tube 1 was inflated until 0.766 kg was measured by the dynamometer corresponding to 2.5 kPa measured with the manometer and representing score 1.

2.4.4 Training of the observers

Nine observers (three male and six female) consented to participate in the study, including three students of veterinary medicine (fourth, fifth, and sixth year), three graduated veterinarians, and three milkers. First, the four inflated tire tubes were presented one by one in a random order to train palpation and the use of the dynamometer, respectively. Every observer had to palpate the tubes until four consecutive presented tire tubes (i.e., all four scores) were scored correctly. The use of the dynamometer and the guidelines of the SOP were explained and all observers had to measure firmness of all four tire tubes until their results were concordant with the calculated firmness.

2.4.5 Experiment 1

The objective of experiment 1 was to determine the WOR of estimates of udder firmness determined by palpation and the dynamometer. Udder firmness of 25 cows was evaluated on a single day both by palpation with the fingertips and by using a dynamometer.

To evaluate the WOR of udder firmness scores obtained by palpation, three (one student and two milkers) of the nine trained observers were randomly selected. Each of the

three observers obtained one measurement per cow. This procedure was repeated another nine times within 45 min before and within 45 min after milking, resulting in overall 10 measurements per cow and 500 measurements per observer. The observers measured and recorded their results on case report forms independently of each other within 2 ± 1 min. Blank case report forms were used for each measurement run.

To evaluate the WOR of udder firmness determined with the dynamometer, one additional observer measured udder firmness of the same 25 cows consecutively 10 times per cow within 45 min before and within 45 min after milking by using a single dynamometer. As described above, cows were selected by chance and fixed in the head locks.

2.4.6 Experiment 2

To determine the BOR, udder firmness of 100 cows was determined on four different days by all nine observers both by palpation and by using the dynamometer. The procedure of measuring was identical to experiment 1. Due to logistical reasons, udder firmness was recorded first either by palpation (n = 5) or by using the dynamometer (n = 4). In total, four different dynamometers were used.

2.4.7 Statistical analysis

Data were entered into Excel spreadsheets (version 2010; Microsoft Corp., Redmond, WA) and statistical analysis performed with SPSS for Windows (version 20.0; SPSS Inc., Munich, Germany) and R (version R-2.15.2.tar.gz; http://www.r-project.org/; Statistics Department of the University of Auckland, Auckland, New Zealand). A Kolmogorov-Smirnov test was performed to test whether estimates obtained with the dynamometer in experiments 1 and 2 were distributed normally. Because the estimates were not normally distributed, effect of time (before and after milking) on udder firmness was statistically determined by a Wilcoxon signed-rank test.

A Kruskal-Wallis test was performed to test the effect of study day (n = 4) and palpation score on medians of estimates of udder firmness obtained in experiment 2. Repeatability of both methods of estimating udder firmness was calculated separately for measurements taken before and after milking as well as for all data combined, regardless of time of measurement.

To determine the WOR of measurements obtained with the dynamometer by a single observer, the minimum, maximum, median, interquartile range (IQR), and coefficient of variation were calculated for the 25 measured cows individually and combined. To determine the WOR of estimates obtained by palpation and with the dynamometer, intraclass correlations

(ICC; model: two-way; type: consistency) for average (ICCam) and single measures (ICCsm) were calculated. According to Shrout and Fleiss (1979), the ICC was calculated in a two-way mixed model with measures of consistency due to the study design (i.e., each observer out of a fixed set of observers measured each cow with systematic variability due to observers or measures considered to be irrelevant). An ICC of 0 indicated no relationship between the measurements and their coherence could be regarded as essentially random. If the ICC was 1, all measurements showed a perfect correlation. To determine the BOR of measurements of all nine observers obtained with the dynamometer, ICCam and ICCsm (model: two-way; type: consistency) were calculated. To determine the BOR of udder firmness obtained by palpation, ICCam and ICCsm (model: two-way; type: consistency) were calculated.

To evaluate the relationship between the 4-point palpation scoring system and measures obtained with the dynamometer, the Spearman rank correlation coefficient was calculated. Overall, 1,800 paired observations (i.e., 1,800 measurements obtained by palpation and 1,800 measurements obtained with the dynamometer) were used (nine observers, 100 cows, and two replicates).

We calculated ICC to analyze the BOR and WOR of measurements conducted by multiple observers and both continuous and ordinal data for the following reasons. Intraclass correlation values are equivalent to the weighted kappa (Fleiss and Cohen, 1973) often used to assess agreement of measurements for categorical data (Landis and Koch, 1977). Additionally, ICC is applicable to continuous data (Shrout and Fleiss, 1979). Therefore, comparability of both data sets was ensured. Estimates of udder firmness (n = 1,800) obtained by the dynamometer in experiment 2 were reclassified into a 4-point scoring system to generate ordinal data and to allow comparison of correlated ICC.

2.5 Results

2.5.1 Measurements

In total, 6,100 udder firmness measurements performed by palpation and by using the dynamometer were documented in the pilot trial (n = 500), experiment 1 (n = 2,000), and experiment 2 (n = 3,600). Using the dynamometer by applying the SOP took considerably more time than determining udder firmness by palpation. Specifically, the mean time needed to measure udder firmness of a single cow by palpation and with the dynamometer was 12 $\pm$ 1.2 s and 48 $\pm$ 18 s (P = 0.04), respectively.

2.5.2 Pilot trial

Minimum and maximum udder firmness of the 25 cows enrolled in the pilot trial measured with the dynamometer are summarized in table 1. Median (and IQR) of udder firmness was 1.296 kg (0.947 to 2.355 kg) and 0.572 kg (0.465 to 0.782 kg) before milking and after milking, respectively. Median milking-induced decrease [i.e., difference (and IQR)] in udder firmness was 0.763 kg (0.426 to 1.570 kg; $P < 0.001$). Graduated veterinarians and milking personnel needed an average of three replicates, whereas the students needed an average of two replicates to correctly diagnose the firmness of all four tire tubes.

2.5.3 Experiments 1 and 2

Twenty-five and 100 cows were enrolled to determine WOR and BOR of measuring methods in experiments 1 and 2, respectively. Estimates of udder firmness measured with the dynamometer by all observers in experiments 1 and 2 ranged from 0.302 to 5.309 kg. The median (and IQR) udder firmness was 1.373 kg (0.996 to 1.845 kg) and 0.659 kg (0.507 to 0.905 kg) before and after milking, respectively. The median milking-induced decrease [i.e., difference (and IQR)] in udder firmness was 0.605 kg (0.350 to 1.017 kg; $P < 0.001$).

To enable comparison of udder firmness values measured in this and a previous study, median udder firmness of cows > 304 DIM (n = 5) was calculated. The median (and IQR) udder firmness of this subset of cows was 0.840 kg (0.604 to 1.135 kg) and 0.443 kg (0.363 to 0.506 kg) before and after milking, respectively. Medians of measurements obtained with the dynamometer in experiment 2 differed ($P < 0.001$) between the four days of the study (figure 1) as well as between different palpation scores (figure 2).

The coefficient of variation of repeated measures obtained with the dynamometer by a single observer (experiment 1) was 9.1% (mean $\pm$ SD: 1.176 $\pm$ 0.107 kg; n = 500). Within-observer repeatability of the same measures of udder firmness was 0.997 (ICCam, 95% CI: 0.996 to 0.998) and 0.971 (ICCsm, 95% CI: 0.959 to 0.981) for all measures (n = 500). Within-observer repeatability of these measures was 0.996 (ICCam, 95% CI: 0.993 to 0.998) and 0.960 (ICCsm, 95% CI: 0.935 to 0.978) before and 0.996 (ICCam, 95% CI: 0.993 to 0.998) and 0.957 (ICCsm, 95% CI: 0.930 to 0.977) after milking. Within-observer repeatability for measures of udder firmness obtained by palpation via three observers was 0.968 (ICCam, 95% CI: 0.960 to 0.975) and 0.753 (ICCsm, 95% CI: 0.706 to 0.798) for all measures (n = 500). Within-observer repeatability of the same measures was 0.931 (ICCam, 95% CI: 0.905 to 0.952) and 0.575 (ICCsm, 95% CI: 0.489 to 0.666) before and 0.942 (ICCam, 95% CI: 0.920 to 0.960) and 0.618 (ICCsm, 95% CI: 0.535 to 0.704) after milking. Between-observer repeatability of measures obtained with the dynamometer by all nine observers of experiment 2 was 0.898 (ICCam, 95% CI: 0.867 to 0.925) and 0.329 (ICCsm, 95% CI: 0.266 to 0.407) for

all measures (n = 1,800). Between-observer repeatability of the same measures was 0.894 (ICCam, 95% CI: 0.860 to 0.922) and 0.483 (ICCsm, 95% CI: 0.405 to 0.569) before and 0.884 (ICCam, 95% CI: 0.846 to 0.915) and 0.458 (ICCsm, 95% CI: 0.379 to 0.545) after milking. Between-observer repeatability of measures obtained by palpation via all nine observers was 0.932 (ICCam, 95% CI: 0.917 to 0.945) and 0.603 (ICCsm, 95% CI: 0.550 to 0.657) for all measures (n = 1,800). The calculated BOR of the same measures was 0.905 (ICCam, 95% CI: 0.874 to 0.930) and 0.513 (ICCsm, 95% CI: 0.435 to 0.597) before and 0.835 (ICCam, 95% CI: 0.781 to 0.879) and 0.359 (ICCsm, 95% CI: 0.284 to 0.447) after milking.

After reclassification of estimates of udder firmness (n = 1,800) obtained by the dynamometer in experiment 2, the recalculated BOR was 0.391 (ICCsm, 95% CI: 0.336 to 0.452) and 0.852 (ICCam, 95% CI: 0.820 to 0.881).

2.5.4 Relationship between two methods of measuring udder firmness

Spearman rank correlation coefficients quantifying the relationship between 900 udder firmness estimates per study day (n = 4) obtained by palpation and measured with the dynamometer were 0.49, 0.58, 0.56, and 0.55 on day 1, 2, 3, and 4, respectively. On all days, a difference ($P < 0.001$) between the two measurement methods existed.

Overall, the Spearman rank correlation coefficient was 0.54 ($P < 0.001$; n = 3,600; figure 2). The coefficient of correlation between the two methods to determine udder firmness was lower before (r = 0.24; $P < 0.001$; n = 1,800) than after milking (r = 0.32; $P < 0.001$; n = 1,800).

Table 1. Comparison of the 4-point palpation system with estimates of udder firmness measured with the dynamometer (n = 500) and tire pressures measured with a manometer in the pilot trial

	Dynamometer (kg)			
Score	Minimum	Maximum	Mean	Manometer (mbar)
1	0.306	1.225	0.766	25
2	1.226	2.144	1.685	125
3	2.145	3.062	2.604	200
4	3.063	3.981	3.523	250

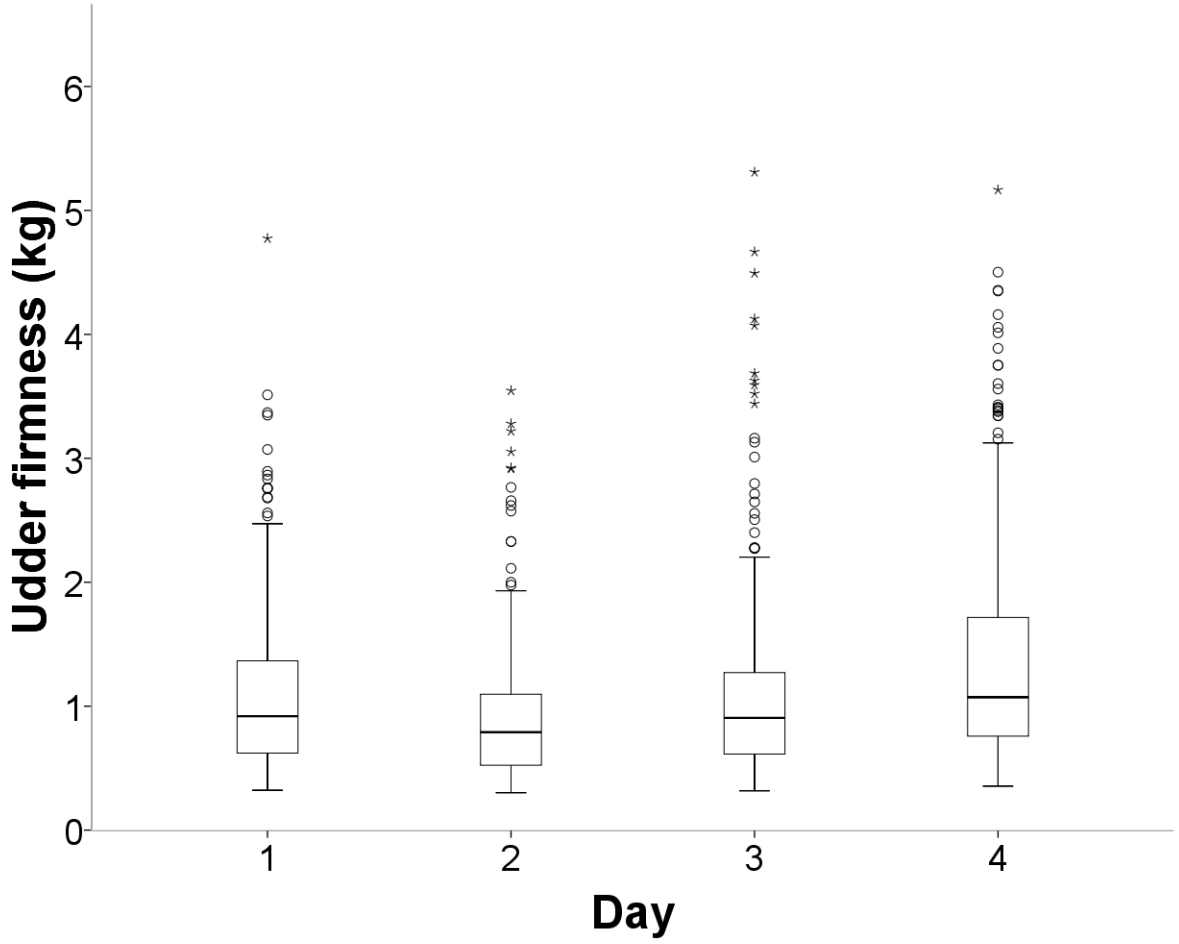

Figure 1. Boxplot of estimates of udder firmness (kg) measured with the dynamometer on day 1 to 4 in experiment 2. ○ = outlier, * = extreme value

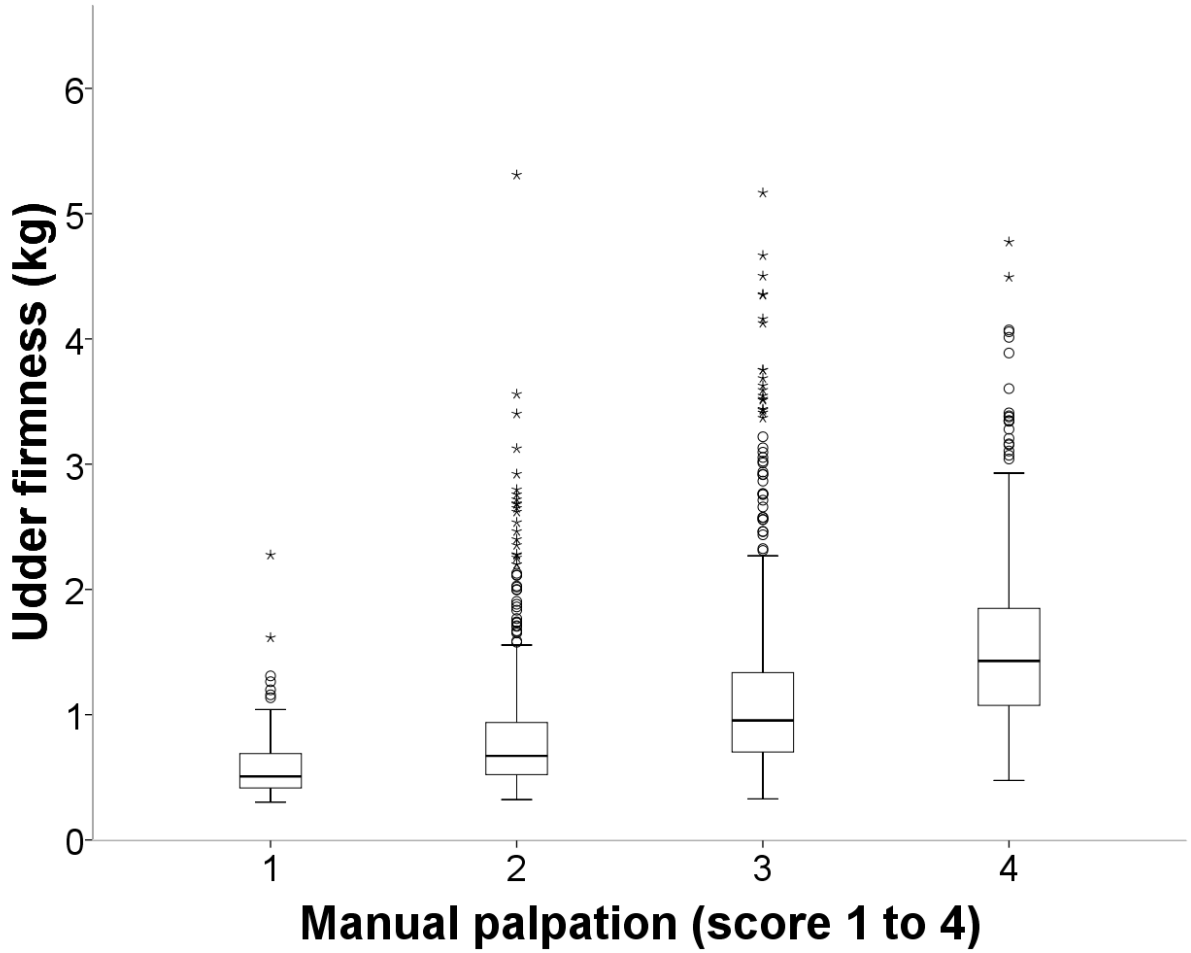

Figure 2. Boxplot of relationships between estimates of udder firmness obtained by palpation (palpation score 1 to 4) and with the dynamometer (kg). ○ = outlier, * = extreme value

2.6 Discussion

2.6.1 Measurements

We assumed that a validated technical device (i.e., dynamometer) would produce less subjective values than palpation. Therefore, we used the dynamometer as a reference device to examine if an objective and repeatable estimate of udder firmness by palpation could be established.

Whereas in a previous study 9.3% of the measurements with the dynamometer exceeded the cut point of 10% coefficient of variation and were regarded as invalid (Bertulat et al., 2012), in the current study, 24% of the measurements had to be repeated. We speculated that the high number of measurements conducted on a given day (n = 50 to 62) and the considerable time commitment (2.5 h per day of study) by each of the observers might have decreased SOP compliance and caused a higher repetition rate.

Previous studies implementing palpation of udder tissue to detect signs of inflammation did not provide any details of the method of palpation or the location within the udder (Polat et al., 2010; Petrovski et al., 2011). Other studies conducting udder firmness measurements and applying a firmness scoring system (Gleeson et al., 2007; O'Driscoll et al., 2011) did only vaguely describe the location of palpation (i.e., between the hind legs). Houe et al. (2002) compared clinical evaluations of udder health characteristics among observers using a defined scoring system and the palpation method described by Rosenberger et al. (1990). Those authors quantified the agreement of evaluation of udder hardness via a 5-point score (i.e., categorical data by calculating above-mentioned κ and weighted κ values). They compared recordings of observers two by two and found poor agreement between clinical parameters not directly related to pathological conditions, such as udder hardness (κ = 0.31), but good agreement for pathological changes such as nodes (κ = 0.7). In their study, palpation encompassed a superficial and deep palpation of every quarter after milking with the palm of the hand, whereas in our study observers determined udder firmness just with their fingertips at the same point at which the dynamometer was applied. Previously, it has been demonstrated that udder firmness changes considerably between front and hind quarters as well as within the location of a given quarter (Bertulat et al., 2012). To ensure objective and repeatable measurements with the dynamometer, Bertulat et al. (2012) outlined in their SOP that the cow must stand still with all four legs on a level ground during measurement. We observed that a measurement with a coefficient of variation less than 10% could be ensured when following this procedure. The measuring point was located in the horizontal and vertical center of the left hind quarter. To address the objective of our study comparing palpation with a technical device, it was mandatory to conduct the measurements at the same quarter and at

the same level to exclude bias. Therefore, our method of palpation was chosen to enable a more standardized comparison of measurements between observers and between the two methods.

Other variables could have biased the results and decreased repeatability and correlation between the two methods. Before milking, the weight of the milk expanded the system of udder suspension, which most likely affected udder firmness. Discomfort and pain has been described for the cow lying with a filled udder due to external pressure on the udder (Österman and Redbo, 2001). We speculate that the position of hind legs relative to the hind quarter could also affect udder firmness due to increasing external pressure on the udder. Because we did not lift the udder quarter while estimating udder firmness, our palpation method probably was more susceptible to changes in udder firmness due to higher tension of the udder suspension system before milking or the position of the hind legs and ankle joints exerting external pressure onto the udder, as described previously (Österman and Redbo, 2001).

2.6.2 Pilot trial

A wide range of udder firmness was determined by the 500 conducted measurements. Minimum values of udder firmness obtained with the dynamometer were almost identical in the pilot trial and the experiments. This may be due to the lower threshold of 0.300 kg of the dynamometer; but only 3.4% of all values were between 0.300 and 0.400 kg. The maximum values, however, were different between the pilot study and the experiments. This is probably due to the larger sample size of experiment 2 compared with the pilot trial and the larger number of observers. It is unfortunate that data to compare our findings with are not yet available.

As no gold standard exists for the diagnosis of udder firmness, the objective of the in-vitro trial was to generate a reference standard having properties (size and firmness) similar to an udder quarter. Except for isolated perfused bovine udders used in pharmacodynamic studies (Ehinger et al., 2006; Kietzmann et al., 2008, 2010), in-vitro approaches establishing defined reference standards to investigate characteristics of diagnostic methods have not been described. Other studies, however, mentioned the lack of a gold standard for udder health (Houe et al., 2002) and for udder examination and found evidence that the sensitivity and specificity of diagnosing CM differ among observers (McDougall, 1999). By evaluating different materials such as foams, rubbers, and inflatable objects, we found that the pressure of an inflated tire tube could be adapted to represent a certain firmness and measured accurately, thus creating an artificial reference for udder firmness. This allowed training of multiple observers with specimens of identical characteristics and to study whether the described

method of palpation is adequate to categorize firmness on a 4-point classification system. We used an even-numbered score to avoid biased results due to a middle option of an odd-numbered score (e.g., point 3 of a 5-point score; Clark and Watson, 1995). Additionally, the range of udder firmness was suitable for a 4-point score. An increase in score points (e.g., 6-point palpation scoring system) could have led to reduced validity (Clark and Watson, 1995). It is noteworthy that after only a few replicates, each observer was able to correctly classify the four firmness levels. It is obvious that the complex anatomy of a mammary gland could not be perfectly simulated, but an in-vitro model with a reference standard that could be described specifically (i.e., pressure applied in kilopascals) was necessary to evaluate and standardize palpation.

2.6.3 Observers

The professional background (students, veterinarians, and milkers) and an assumed different experience did not affect the ability to classify the four different firmness levels by palpation. In vitro, only two to three attempts were needed to categorize four firmness levels correctly in the 4-point classification system. We speculate that a training effect resulted from the possibility of comparing all different firmness levels in a short time and directly one after another. In vivo, the Spearman rank correlation coefficient did not change over the days of the trial. Therefore, we did not observe an improvement of the correlation between estimates of udder firmness obtained by palpation and measurements obtained with the dynamometer over time. This observation differed from the results of an earlier study (Houe et al., 2002) that found increased κ correlation values for examinations conducted on different days. Those authors assumed this to be an effect of practicing clinical examination. In their study, however, only sense-based udder examinations (i.e., inspection and palpation) were conducted, whereas in our study, a technical device (i.e., dynamometer) was compared with palpation. We assume that improvement of correlation over time can be realized when sense-based and, therefore, trainable methods are used. The observers in their study were given a vivid description of the scoring system, but training was not conducted before the initiation of the study. We speculate that in our study training could have contributed to a more consistent diagnostic performance, reducing variation that might have effected correlation. Interestingly, as a result of their findings Houe et al. (2002) stated that a need seems to exist for an increase in training and calibration of score values.

2.6.4 Repeatability of estimates of udder firmness in experiments 1 and 2

We are aware of potential criticism regarding the use of the coefficient of variation and ICC in the context of non-normally distributed data. However, we chose ICC instead of weighted κ to facilitate comparison of results. Additionally, we suggest a mixed model to be an appropriate statistical method for future studies due to the possibility of estimating the contributions to variability from cows, for example.

2.6.5 Dynamometer

The measurements were conducted at the same time of the day and within 45 min before and after milking in each replicate. We assume that this consistency in time contributed to repeatability of estimates of udder firmness. This finding substantiates the recommendation of a previous study conducted by our group (Bertulat et al., 2012). Significantly different medians of measurements (figure 1) were obtained with the dynamometer in experiment 2 simply because different animals were used on the four days of the study.

Our results indicate that measurements obtained with the dynamometer by a single observer were highly repeatable. Whereas averaged measurements of different observers also had high repeatability, a single measurement of one observer was poorly related to the measurements of other observers. These data are contrary to a previous study (Bertulat et al., 2012) in which a high BOR with an average inter-observer variation for all measurements of 11.3% ($r = 0.94$) was described. The different statistical calculations (i.e., comparing three pairs of observers separately vs. ICC), a smaller number of observers (two vs. nine), or biasing conditions (movement of cows between measurements and variation in ankle joint position) can help explain this discrepancy. As in our study, Tucker et al. (2007, 2009) described a standardized measuring point and defined the penetration depth for the device used. Repeatability, however, was not calculated and udder firmness values were obtained with a different measuring device, resulting in values with a different unit (i.e., gram of force). Therefore, our estimates of udder firmness are not directly comparable with their study. Bertulat et al. (2012) validated the same device as used in our study and applied an SOP, but did not mention absolute udder firmness values. In a previous trial, we enrolled only cows within the last week before dry-off (343 $\pm$ 39 DIM) and until nine days after dry-off to study udder firmness after dry-off (Bertulat et al., 2013). Due to the different DIM and higher daily milk yield in the current study, data of the two studies are also not directly comparable. Cows > 304 DIM ($n = 5$) in the current study had very similar average udder firmness before and after milking to the baseline values of cows before dry-off in a previous study (Bertulat et al., 2013).

2.6.6 Palpation

Categorizing udder firmness by palpation on a 4-point scale was highly repeatable for a given observer both for a single or average measurement. Averaged measurements of different observers showed high repeatability and single measurements between observers were moderately correlated. These results indicate that a single classification of udder firmness on a 4-point scale obtained by palpation, carried out once and conducted by different observers, is more comparable than measurements obtained by the dynamometer by different observers. Between-observer repeatability of average measurements of both measuring methods was similar.

Even though ICC was calculated for both variables, it must be noted that estimates of firmness obtained by palpation were on an ordinal scale (1 to 4) and values obtained by the dynamometer were continuous variables. A direct comparison of ICC using different data scales, however, is critical. Therefore, we reclassified estimates of udder firmness (n = 1,800) obtained by the dynamometer in experiment 2 into a 4-point scoring system to generate ordinal data and to allow a comparison of correlated ICC. The recalculated interclass correlation remained similar to the first calculated correlation, still indicating a better correlation for a single measurement obtained by palpation and different observers.

In contrast to our results, a previous study (Houe et al., 2002) demonstrated poor agreement of udder examination when classifying non-pathological conditions, such as hardness of the udder parenchyma. This observation was based on 178 paired observations of clinical evaluations performed by five clinicians in four herds within 90 min after milking. A 5-point scale was used to classify hardness of udder tissue (1 = soft, 3 = firm, and 5 = hard). The calculated κ value for the parameter udder hardness was 0.31, indicating low repeatability. This discrepancy can be explained by the different methods used to determine udder firmness or hardness. Whereas observers in our study used their fingertips and indented the udder tissue at identical locations, a palpation of the whole quarter and udder was conducted in the study of Houe et al. (2002). We speculate that quarters with inhomogeneous tissue composition will cause different findings. Our results demonstrate that studies working with more than one observer to perform udder examination could be biased and of low informative value. Additionally, poor observer agreement could lead to false conclusions regarding dairy herd management and therapy of udder diseases due to a lack of a standardized decision basis.

2.6.7 Relationship between two methods of measuring udder firmness

It must be mentioned that comparison of two measurement methods via calculation of a correlation coefficient could be critical for several reasons. It has been described that

sensitivity of correlation methods to sample heterogeneity could result in wrong conclusions regarding the agreement of measurements (Bland and Altman, 1995; Atkinson and Nevill, 1997). Additionally, correlation coefficients were described as a measure of association, not a measure of agreement (Altman and Bland, 1983). In our study, an ordinally scaled 4-point palpation scoring system was compared with continuous-scale dynamometer values. We used the Spearman rank correlation coefficient to assess the relationship between both methods of measuring udder firmness. We are aware of the limitations of the correlation coefficient as a measure of agreement (Altman and Bland, 1983), but the scale of our data limited use of more adequate methods. A comparison of results with other studies was not possible simply due to a lack of publications investigating a similar approach.

Association of estimates of udder firmness on the 4-point scoring system obtained by palpation and with the dynamometer was moderate. Although medians of estimates of udder firmness differed significantly between the four classes of the palpation scoring system, the boxes and whiskers of the box plots overlap (figure 2). Therefore, it is not possible to classify an udder into one specific score by measuring firmness in kg and vice versa. Besides the subjective nature of palpation, one explanation for the poor relationship could be movements of the cow between measurements leading to different postures of the hind legs relative to the udder and influencing udder firmness. Furthermore, when comparing two methods, it is important to recognize that neither approach may be ideal. Nevertheless, data of our and a previous study (Houe et al., 2002) provide evidence that palpation of the udder is a method with limited repeatability when multiple observers are involved. It is noteworthy that studies comparing multiple diagnostic methods to examine udder health are scarce.

Correlation between palpation and dynamometer before milking was lower than the correlation after milking. We assume that some of observers were reluctant to press the measuring tip of the dynamometer or their fingertips with adequate pressure into the tissue of hard udders found before milking, thus confounding the measurements before milking. Additionally, the volume of the milk or the weight effect on the udder suspension system could have affected measurements. Our data support the recommendation of an udder examination after milking and provide science-based information for the descriptions in textbooks (Rosenberger et al., 1990; Radostits et al., 2001) in which a palpation after milking was advised.

In our study, all measurements were conducted using healthy udders to exclude additional bias through inhomogeneous swelling. Considering pathological conditions, further research is warranted to understand how udder firmness develops in infected udders.

2.7 Conclusions

Our results demonstrate that estimates of udder firmness generated by palpation and with the dynamometer were lowly related. Although observers were trained in palpation and scoring udder firmness on a 4-point scale and in the use of the dynamometer before determination of udder firmness in vivo, correlation was limited. Udder firmness in dairy cows can be measured repeatably by palpation and with the dynamometer, especially when performed by a single observer. Although imperfect, a 4-point palpation scoring system provides a feasible and easy-to-use classification system to estimate udder firmness.

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3 ADDITIONAL UNPUBLISHED WORK

Udder firmness as a possible indicator for clinical mastitis

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For the sake of consistency, the additional contribution is formatted in an identical style as both research papers.

3.1 Abstract

Swelling of the mammary gland is an important sign to detect clinical mastitis (CM) in dairy cows. The overall objective of this study was to evaluate if udder firmness can be used as a cow-side indicator for mastitis and to evaluate how CM affects firmness for 14 days after diagnosis. A dynamometer was used to objectively determine udder firmness before and after milking in 45 cows with CM and 95 healthy cows. Udder firmness of both hind quarters was measured daily on three locations (upper, middle, lower measuring point) from the day of mastitis diagnosis until day 7 and again on day 14.

Firmness of the middle measuring point was highest before and after milking in all cows. Udder firmness before milking was similar in quarters without and with CM. Subsequently, we concentrated on firmness measured on the middle point after milking. After milking, quarters with CM were firmer than healthy quarters. An increase of firmness of a quarter with mastitis did not affect firmness of the healthy neighboring quarter nor did firmness of all healthy quarters differ. One firmness value per cow i.e., Δ firmness (difference in udder firmness between both hind quarters) was used for all further calculations. In all cases, CM affected Δ firmness. In cows in their second and greater parity, Δ firmness was also affected by milk yield per day and DIM.

The threshold for detection of CM using Δ firmness was 0.282 kg (area under the curve: 0.722; sensitivity: 64.3%, specificity: 89.7%) and 0.425 kg (area under the curve: 0.817; sensitivity: 62.5%, specificity: 96.7%) in first-parity cows and older cows, respectively.

Cows with CM had a higher Δ firmness compared to cows without CM throughout the 14 days after the mastitis diagnoses. Parity had an effect on Δ firmness. Depending on systemically signs of sickness, mastitic cows were divided into cows mild to moderate (n = 21) or severe mastitis (n = 24). Bacteriological cure was defined based on two milk samples taken at 7 and 14 days after enrollment. Cows with severe mastitis suffered from a firmer udder on all measuring days. An effect of parity and bacteriological cure on Δ firmness did not exist.

Cows not clinically cured showed an increased Δ firmness of 0.560 kg compared to cured cows.

In conclusion, udder firmness can be a useful indicator for CM. Further research is warranted to evaluate if udder firmness can be used as a predictor for the prognosis of a CM or the cure of inflammation.

3.2 Key words

udder firmness, dynamometer, clinical mastitis, diagnosis

3.3 Introduction

Mastitis is a highly relevant disease (Hertl et al., 2011; Hertl et al., 2014) and the most common indication for the use of antimicrobial agents in dairy cows (Thomson et al., 2008). Approximately 50% of all parenteral administered antibiotics are used for the therapy of clinical mastitis (CM; Pol and Ruegg, 2007). A prudent use of antibiotics, however, has been emphasized and advocated as the issue is a top priority public health challenge (e.g., Oliver et al., 2011, Machado et al., 2014).

Detection of the infected quarter precedes an antibiotic therapy of CM (Oliver et al., 2011). Clinical symptoms to detect CM include changes in milk characteristics and redness, swelling and warming of the infected quarter. Besides checking the milk for abnormalities, determination of udder firmness is a plausible and practical method to diagnose this disease. Veterinarians and farmers frequently base treatment decisions on clinical symptoms of the udder (Swinkels et al., 2015). Udder firmness, however, seems to be a very difficult variable to determine correctly (Fossing et al., 2006). Also, there are no data available to quantitatively define a healthy udder using specific thresholds for udder firmness. Besides descriptions in textbooks (Rosenberger et al., 1990; Radostits et al., 2001), data are not available to objectively differentiate healthy from affected udders. Additionally, most recently farmers' insecurity in mastitis therapy and wrong decisions regarding extended treatment of CM has been described (Swinkels et al., 2015). Therefore, more research is warranted on the evolution of clinical criteria (Swinkels et al., 2015) and specific guidelines to provide differentiation between cows without and with CM are needed. The timely detection of signs of CM would also allow shorter and more effective drug treatments (Trevisi et al., 2014).

It was demonstrated (Bertulat et al., 2012; Rees et al., 2014) that udder firmness in healthy cows could be determined with a good repeatability by trained observers by using an electronic handheld device (i.e., a dynamometer). Furthermore, preliminary results indicated that the milking-induced decrease of udder firmness was lower in cows with CM compared to cows without CM (Rees et al., 2013). In the latter study, however, cows with CM were included on different days relative to the onset of infection. Thus, different stages of CM were included confounding the results.

Therefore, the overall objective of this study was to evaluate if udder firmness can be used as a cow-side indicator for mastitis. Specifically, we set out to 1) establish firmness thresholds for the differentiation between cows without and with CM, and 2) to evaluate how CM affects udder firmness within 14 days after diagnosis.

3.4 Materials and methods

3.4.1 Housing and animals

The study was conducted from April to August 2014 on a commercial dairy farm milking 1,200 Holstein-Friesian dairy cows in Sachsen-Anhalt, Germany. Cows were housed in a free stall barn with slatted floors and cubicles equipped with rubber mats. Cows were fed a TMR consisting of 38.5% corn silage, 35.9% concentrate mineral mix, 22.5% grass silage, and 3.1% barley straw. Feed was delivered via a conveyer belt system 10 times per day. All cows had ad libitum access to water. Cows were milked three times a day during three milking shifts from 0700 to 1400 h and 1500 to 2200 h and 2300 to 0600 h in a 52-stall external rotary milking parlor (Lemmer-Fullwood GmbH, Lohmar, Germany). Milk yield per cow and milking was displayed. The average 305-days milk yield was 10,147 kg (4.04% fat and 3.35% protein). During the study period, average SCC was 250,000 per ml bulk tank milk and incidence of CM was 24 cases per 100 cow-years.

3.4.2 Mastitis management

The milking personnel checked all cows before each milking for signs of CM by visually examining foremilk on a dark surface as the standard procedure. An udder quarter was diagnosed as having CM when clots or flakes in foremilk samples were observed. Based on a severity classification system described previously (Oliveira et al., 2013) such a case was defined as a mild to moderate case of CM. Because *Streptococcus uberis* was known to be the dominant pathogen causing CM on this farm, the infected quarter was treated with an intramammary infusion of 3,000,000 IU procaine benzyl penicillin (Procain-Penicillin-G Injektor aniMedica 300 mg/ml; Selactavet, Weyarn-Holzolling, Germany) every 24 h for three consecutive days. When signs of generalized sickness such as reduced feed intake, dullness, or a rectal temperature above 39.5°C were present, the case was considered severe (Oliveira et al., 2013) and intramammary treatment was complemented by parenteral administered antibiotic and non-steroidal drugs (NSAID). More precisely, 10,000 IU penethamate hydriodide (Mamyzin; Boehringer Ingelheim GmbH, Ingelheim, Germany) and 2.0 mg marbofloxacin (Odimar 100mg/ml; Animalcare Limited, Dunnington, United Kingdom) or 0.5 mg meloxicam (Melovem 20 mg/ml; Dopharma Research B.V., Raamsdonksveer, the Netherlands) or 2.2 mg flunixin meglumine (Finadyne; MSD Animal Health GmbH, Luzern, Switzerland) per kilogram of body weight were administered intramuscularly or intravenously in the latter case. All treatments were documented in the on-farm computer program (Herde; Agrosoft, Paretz, Germany).

Cows with signs of CM diagnosed by the milking personnel were moved to the mastitis pen. They were reintroduced into the production groups after the withdrawal period for milk

and the appearance of the milk returned to normal. Cubicles in the mastitis pen were equipped with rubber mats and covered by a 10 cm layer of recycled manure solids from the on-site biogas plant. All cubicles were cleaned manually three times a day and the layer was topped once a week.

3.4.3 Sample size and enrollment

Sample size calculation was performed with WinEpiscope 2.0 using a 95% CI and 80% of power (Thrusfield et al., 2001). The minimum number of cows to be included was estimated based on previously estimated mean udder firmness after milking (Rees et al., 2013) of cows without (0.700 kg) and with CM (1.300 kg). A two-sided test was selected with an α of 0.05 and a power (1 − β) of 0.80. A minimum sample size of 38 cows per group was calculated.

Cows were enrolled when the milking personnel observed CM in one of the hind quarters. Day of enrollment was considered as day 0 (D0). Quarter milk samples were collected immediately before milking from both hind quarters. Six measuring points were marked and udder firmness was determined as described below. Also, rectal temperature was measured and actual milk yield recorded. Udder morphology (i.e., the length from the teat base to the rear udder attachment and the width of hind quarters; figure 1) was determined using a measuring tape. To create an appropriate negative control group, two randomly selected cows entering the milking parlor within 10 min after the cow with CM were enrolled and processed the same way. These two cows had to be free from signs of CM. After milking, the cow with CM was moved to the mastitis pen while the cows without CM remained in their original group.

3.4.4 General procedures and sampling

Udder firmness was determined by using a dynamometer (Penefel DFT 14; Agro Technologies, Forges-les-Eaux, France). In total, four dynamometers were used following the standard operation procedure described by Bertulat et al. (2012). All dynamometers were validated before the field trial started.

To quantify effects of location within a given quarter and between quarters, the measuring points were located in the upper, middle and lower third of both hind quarters (figure 1). These points were marked with livestock paint crayons (Raidex, Dettingen, Germany) to ensure a consistent measurement location within the udder during the whole study period. The cow had to stand with all four legs on a level surface during the whole measurement. After five consecutive measurements performed within 10 s the dynamometer displayed the arithmetic mean and coefficient of variation. Values with a coefficient of variation exceeding 10% were discarded and the measurement repeated.

Quarter milk samples were aseptically collected immediately before routine milking following procedures described by the National Mastitis Council (Hogan et al., 1999). Milk samples were cooled down immediately and transported to a commercial milk laboratory within two days.

3.4.5 Laboratory procedures

Milk samples submitted to the laboratory were cultured using standard microbiological methods (Hogan et al., 1999). Briefly, a volume 0.01 mL milk was inoculated on esculin blood agar (Oxoid Deutschland GmbH, Wesel, Germany) and the plate was incubated at 37°C. Plates were examined for growth at 24 and 48 h. Bacteria were identified by colony morphology and gram stain. For gram-positive cocci, catalase tests were performed to distinguish catalase-negative *Staphylococcus* spp. from catalase-positive *Staphylococcus* spp. Streptococci were differentiated by using a commercial test kit (Patho Dxtra Strep Grouping Kit; Oxoid Deutschland GmbH, Wesel, Germany) and growth on esculin blood agar plates. Catalase-positive gram-positive cocci were further identified using a coagulase test and hemolysis patterns. Gram-positive bacilli were further identified using the catalase test and biochemical reactions as needed. Gram-negative bacilli were identified by the oxidase test and the EnteroPluritest (Becton, Dickinson and Company, Heidelberg, Germany). Contaminated samples were defined as a mixture of at least three environmental type organisms without isolation of a major mastitis pathogen. The SCC analysis of each quarter milk sample was performed using a DeLaval cell counter (DeLaval GmbH, Glinde, Germany).

3.4.6 Experimental design

Each cow was followed up daily until day 7 (D7) after CM was diagnosed and examined again on day 14 (D14). On all measuring days, udder firmness and rectal temperature were determined and actual milk yield recorded. Additionally, the milking personnel checked the milk of all cows for abnormalities. Udder firmness was assessed in the barn 1.4 $\pm$ 1.2 h before milking and immediately after milking while cows still being in the milking parlor. When a cow was enrolled in the first, second or third milking shift, all subsequent measurements for this cow were also conducted during the first, second or third shift, respectively. Additionally, on D7 and D14, measurements of udder morphology were determined after milking and milk samples collected as described for D0.

3.4.7 Clinical and bacteriological cure

Bacteriological and clinical cure were defined as previously described (Schukken et al., 2013). In brief, a quarter that was infected at the beginning of treatment was defined as bacteriologically cured when the organism that was identified in the milk sample on D0 was not present in the samples of D7 and D14. Clinical cure was defined as the presence of normal milk on D7 and D14.

3.4.8 Data processing and statistical analysis

Data were entered into Excel (version 2010; Microsoft, Redmond WA, United States) and statistical analysis performed with SPSS for Windows (version 20.0; SPSS Inc., Munich, Germany) and MedCalc (version 12.5.0.0; MedCalc software, Mariakerke, Belgium). Statistical significance was set at $P < 0.05$ and a trend for significance was set at $P < 0.10$. All data were tested for normal distribution via visually examination of histograms.

Data regarding parity (first or second and greater parity), gram-staining characteristics (gram-negative, gram-positive, bacteriologically negative) were categorized. Udder morphology values were used to define a symmetric (i.e., difference in width and length between both hind quarters < 2cm) or asymmetric (difference in width and length between both hind quarters $\geq$ 2 cm). The SCC values were $\log_{10}$ transformed.

Udder firmness before and after milking as well as milking-induced decrease in udder firmness in cows without and with CM was compared using a Wilcoxon signed-rank test. We first compared firmness within a given quarter (i.e., firmness within the three different locations) measured before and after milking using a Friedman test to reduce clustering of data on the quarter level and to detect possible influence of the location on firmness within a given quarter. A Wilcoxon signed-rank test was assessed to further compare firmness of the middle measuring point with firmness of the upper and lower measuring point, respectively. The results of the latter calculations indicated that udder firmness measured at the middle measuring point was least variant. Therefore, we used the middle measuring point and udder firmness after milking for all subsequent calculations. To establish firmness thresholds (i.e., first objective), only values measured on D0 were used for the comparison between cows without and with CM. We used non-parametric tests because these data were not normally distributed. First, a Wilcoxon signed-rank test was used to compare firmness of both hind quarters within all cows. Second, we compared udder firmness of all healthy quarters of healthy cows using a Kruskal-Wallis test. Third, firmness of all healthy hind quarters of healthy cows was compared with healthy hind quarters of cows with CM using a Kruskal-Wallis test to evaluate the effect of a CM on the firmness of the healthy neighboring quarter. To further reduce data clustering on the cow level (i.e., to have one instead of two firmness values per cow), difference in udder firmness between both hind quarters measured on the middle

measuring point of a given cow (Δ firmness) was calculated and compared between cows without and with CM using a Kruskal-Wallis test.

Influencing factors on udder firmness were tested by generating a generalized mixed model with Δ firmness after milking as the dependent variable. Tested factors were CM (yes, no), continuous milk yield per day, 100-days milk yield, DIM, parity (first, second and greater parity), gram-staining characteristics (gram-negative, gram-positive, bacteriologically negative) and symmetry between hind udder quarters (yes, no). Interactions were tested between CM and DIM, parity and milk yield per day, respectively. The statistical model was built according to the model building strategies described previously (Dohoo et al., 2009; Bertulat et al., 2013). The significance level was set at $P \leq 0.05$.

A receiver operating characteristic (ROC) curve was calculated to compare the diagnostic performance of udder firmness for the detection of CM. Interpretation of ROC curves was based on the area under the ROC curve (AUC) as well as the positive predictive values and negative predictive values. The tested parameter was Δ firmness measured with the dynamometer after milking on D0. The best thresholds were chosen based on the highest sum of sensitivity and specificity. Because results obtained from the mixed model indicated an influence of parity on udder firmness, we further conducted ROC analysis for cows in their first or second and greater parity separately. To guarantee a minimum sensitivity of approximately 70% for a 95% confidence interval, sensitivity of 80% and a specificity of 99% was proposed for automatic in-line CM detection by the Annex of an International Standard ISO/FDIS 20966 (Automatic milking installations - Requirements and testing) of the International Standard Organization (ISO, 2007). For other cow-side tests such as milk temperature, lower sensitivities and specificities of 50 and 70% (Hillerton, 2000) and 77 and 66% (Pohl et al., 2014) were described. To achieve these test characteristics we further calculated thresholds using these values.

To meet our second objective i.e., to evaluate how CM affects udder firmness within 14 days after diagnosis, the effect of CM on udder firmness measured with the dynamometer after milking during the first 14 days after mastitis diagnosis was evaluated in two linear mixed-model ANOVAs. For the first model, Δ firmness was the dependent variable with day (D0 to D7 and D14; n = 9) as the repeated measure. All cows (n = 140) were included. The effect of CM (yes, no), parity (first, second and greater parity), continuous milk yield per day and DIM as fixed factors and the random effect of cow were included in the model. Moreover, the diagonal covariance structure was used. A second model was built to test the influence of severity of CM (mild to moderate CM, severe CM) including only cows with CM (n = 45). Fixed factors were parity (first, second and greater parity), severity (mild to moderate CM, severe CM), bacteriological and clinical cure (yes, no). Models were built according to the model building strategies described previously (Dohoo et al., 2009; Bertulat et al., 2013). Data

regarding Δ firmness were $\log_{10}$ transformed before analysis to achieve normal distribution, but back-transformed values are reported.

Because SCC was highly correlated with CM during the first step of the model building process, we could not include SCC values in the final models. We therefore checked the difference of $\log_{10}$-transformed SCC in quarters without and with CM as the dependent variable on day D0, D7 and D14 in a linear mixed-model ANOVA. All quarters (n = 280) were included. In detail, day (D0, D7, and D14; n = 3) was the repeated measure; CM (yes, no), parity (first, second and greater parity), continuous milk yield per day, and DIM were fixed factors and the random effect of cow were included in this model. The model was built according to the model building strategies described above. Parity and CM where the factors included in the final model.

3.5 Results

In total, 140 cows (21 with mild to moderate CM, 24 with severe CM, 95 without CM) were enrolled. Cows without and with CM were in their first (n = 35 and 13), second (n = 25 and 4), third (n = 21 and 12), fourth (n = 11 and 13) and fifth (n = 3 and 3) parity. Healthy and CM cows were 163 ± 103 and 151 ± 104 DIM (mean ± SD), respectively.

3.5.1 Milk samples, cure, and SCC

We found gram-positive and gram-negative bacteria in 24 and 10 out of 45 quarters with CM, respectively. Eleven quarters were bacteriologically negative. Out of the 45 cows with CM, 21 suffered from mild to moderate CM. In these 21 cows, gram-positive and gram-negative bacteria were found in 11 and 7 cases, respectively. Three samples were bacteriologically negative. This distribution of gram-staining characteristics was similar (P = 0.144; Chi-squared test) in cows with severe CM (n = 24). Specifically, bacteriologically negative samples, gram-positive and gram-negative bacteria were found in 8, 13 and 3 cows with severe CM, respectively.

Cases were only eligible for bacteriological cure when the treated quarter was infected at the time of enrollment into the study. This was the case in 34 out of 45 quarters with CM (76%). Bacteriological cure was observed in 17 of these 34 cases (50%). In detail, 11 out of 21 cows with mild to moderate CM (52%) and 6 out of 24 cows with severe CM (25%) were bacteriologically cured. Clinical cure was observed for 29 out of 45 enrolled cases (64%) and in 67% of the cows with mild to moderate CM and in 63% of the cows with severe CM. Clinical cure was 45% for the quarters without discernible bacteria at enrollment, 46% for gram-positive bacteria, and 60% for gram-negative bacteria.

Log_{10} SCC in quarters with CM (n = 45) was higher ($P < 0.001$) compared to quarters without CM (n = 235) and decreased over time (figure 6). Parity had a negligible effect on SCC ($P = 0.094$).

3.5.2 Udder firmness

Overall range of udder firmness values measured with the dynamometer (n = 7,560) was 0.300 kg to 11.390 kg. Before milking, median udder firmness in quarters without and with CM was 1.867 kg [interquartile range (IQR): 1.243 to 2.671 kg; n = 6,340] and 1.948 kg (IQR: 1.213 kg to 2.819 kg; n = 1,220), respectively ($P = 0.675$). After milking, udder firmness differed ($P < 0.001$) between quarters without (0.636 kg, IQR: 0.514 to 0.783 kg) and with CM (1.036 kg, IQR: 0.704 to 1.702 kg). Median milking-induced decrease of udder firmness was 1.207 kg (IQR: 0.649 to 1.914 kg; $P < 0.001$) in quarters without CM and 0.745 kg (IQR: 0.292 to 1.359 kg; $P < 0.001$) in quarters with CM.

Firmness between the three locations within a given quarter differed ($P < 0.001$) both in quarters without (n = 1,848 and 2,081) and with CM (n = 356 and 399) measured before and after milking, respectively. Before milking, median udder firmness of the upper measuring point was 23.6% (1.825 kg, IQR: 1.260 to 2.570 kg; $P < 0.001$) and 33.1% (1.605 kg, IQR: 1.070 to 2.248 kg; $P < 0.001$) lower, whereas firmness of lower measuring point was 59.4% (1.420 kg, IQR: 0.950 to 2.100 kg; $P < 0.001$) and 30.0% (1.680 kg, IQR: 1.103 to 2.758 kg; $P < 0.001$) lower compared with the middle point in quarters without (2.390 kg, IQR: 1.770 to 3.200 kg) and with CM (2.400 kg, IQR: 1.643 to 3.340 kg), respectively. After milking, median firmness of the upper measuring point was 20.6% (0.573 kg, IQR: 0.464 to 0.699 kg; $P < 0.001$) lower and firmness of the lower measuring point was 13.6% (0.624 kg, IQR: 0.499 to 0.767 kg; $P < 0.001$) lower than the middle point (0.722 kg, IQR: 0.600 to 0.864 kg) in quarters without CM (figure 2). In quarters with CM, median firmness of the upper measuring point was 39.1% (0.791 kg, IQR: 0.588 to 1.135 kg; $P < 0.001$) lower than firmness of the middle measuring point (1.297 kg, IQR: 0.844 to 1.959 kg; $P < 0.001$; figure 2). There was no difference in firmness of the latter to the lower measuring point (1.203 kg, IQR: 0.771 to 2.053 kg; $P = 0.257$; figure 2).

On D0 after milking, firmness of both hind quarters within a cow without CM did not differ ($P = 0.369$). Firmness within a cow with one CM and one healthy quarter, however, differed ($P < 0.001$; figure 3). Median udder firmness of all healthy quarters i.e., all right (0.770 kg, IQR: 0.629 to 0.935 kg; $P = 0.931$; n = 95) and left (0.745 kg, IQR: 0.624 to 0.885 kg; $P = 0.349$; n = 95) healthy quarters of healthy cows was similar after milking (figure 3). There was no difference ($P = 0.419$) in firmness of all healthy quarters of healthy cows (0.755 kg, IQR:

0.629 to 0.908 kg; n = 190) compared to healthy quarters of cows with CM (0.704 kg, IQR: 0.617 to 1.024 kg; n = 45; figure 3).

Median Δ firmness on D0 differed ($P < 0.001$) between cows without (0.098 kg, IQR: 0.030 to 0.216 kg) and with CM (0.756 kg, IQR: 0.170 to 1.914 kg; figure 4).

Gram-staining characteristics, 100-days milk yield and symmetry between hind udder quarters had no effect on Δ firmness after milking. These variables were excluded from the final model, because they resulted in univariate models with $P \geq 0.2$. Interactions between CM and DIM ($P = 0.008$), CM and parity ($P = 0.036$) and CM and milk yield per day ($P = 0.025$) affected udder firmness. Therefore, another model was calculated considering cows without and with CM as well as cows in their first and second and greater parity, respectively. Except CM ($P < 0.001$), other tested factors did not affect Δ firmness after milking using all cows and first-parity cows, respectively. In cows in their second and greater parity, Δ firmness after milking was affected by CM ($P < 0.001$), milk yield per day ($P = 0.001$) and DIM ($P = 0.001$).

Results of a ROC analysis to determine thresholds for Δ firmness delivering the best combination of sensitivity and specificity in order to differentiate healthy from mastitic cows are in table 1. Thresholds calculated for Δ firmness to achieve ideal sensitivity of 80% (ISO, 2007) by using our data and visual judgement of foremilk as the gold standard were > 0.149 kg for all cows, > 0.020 kg for first-parity cows and > 0.153 kg for older cows (table 1). For an ideal specificity of 99% (ISO, 2007) one identical threshold of > 0.831kg should be used for all cows, first-parity cows and for older cows, respectively.

An effect of CM on udder firmness after milking was observed throughout the first 14 days after mastitis diagnosis. Cows with CM had a higher Δ firmness after milking compared to cows without CM ($P < 0.001$; table 2) on all days. Besides the effect of CM ($P < 0.001$), Δ firmness was affected by parity ($P = 0.016$). An effect of milk yield ($P = 0.960$) or DIM ($P = 0.513$) on udder firmness was not observed. Cows suffering from severe CM had a Δ firmness, which was 2.220 kg higher ($P < 0.001$) compared to cows with mild to moderate CM (figure 5). Cows not clinically cured showed a higher Δ firmness of 0.560 kg compared to cured cows ($P < 0.001$). An effect of parity ($P = 0.140$) and bacteriological cure ($P = 0.262$) on udder firmness did not exist.

Table 1. Diagnostic test characteristics for thresholds of difference of udder firmness between both hind quarters of a given cow in kg (Δ firmness) to identify cows with clinical mastitis measured by a dynamometer considering all cows (n = 140), cows in their first (n = 48) and cows in their second and greater parity (n = 92)

	Enrolled cows		
Test characteristics	All cows	First-parity cows	Second- and greater-parity cows
Threshold of Δ firmness (kg)	0.425	0.282	0.425
AUC[1]	0.809	0.722	0.817
Sensitivity	60.00	64.29	62.50
Specificity	97.89	89.74	96.67
Positive predictive value	93.1	69.2	90.9
Negative predictive value	83.8	87.5	82.9

[1]AUC = Area under the receiver operating characteristic curve

Table 2. Least squares means (and 95% CI) of difference in udder firmness between both hind quarters (kg) in cows without (n = 95) and with (n = 45) clinical mastitis measured with a dynamometer after milking on a measuring point located in the horizontal and vertical center of each udder quarter[1]

Day of study	Clinical mastitis		*P*-value[2]
	No	Yes	
D0	0.073 (0.056 to 0.096)	0.445 (0.263 to 0.751)	<0.001
D1	0.079 (0.063 to 0.096)	0.596 (0.392 to 0.906)	<0.001
D2	0.083 (0.065 to 0.106)	0.363 (0.218 to 0.606)	<0.001
D3	0.066 (0.051 to 0.087)	0.469 (0.300 to 0.731)	<0.001
D4	0.053 (0.040 to 0.070)	0.410 (0.244 to 0.689)	<0.001
D5	0.066 (0.049 to 0.089)	0.462 (0.292 to 0.731)	<0.001
D6	0.067 (0.052 to 0.086)	0.551 (0.353 to 0.862)	<0.001
D7	0.076 (0.061 to 0.094)	0.499 (0.341 to 0.731)	<0.001
D14	0.084 (0.066 to 0.180)	0.340 (0.218 to 0.530)	<0.001
P-value[3]	0.182	<0.001	-

[1]Values are from a linear mixed-model ANOVA accounting for fixed effects of clinical mastitis and parity and a random effect of cow with day as the repeated measure. Values were $\log_{10}$ transformed, but back-transformed least squares means (95% CI) are presented here

[2]*P*-value for comparison of udder firmness in healthy cows and cows with mastitis on a given study day

[3]*P*-value for comparison of udder firmness on all study days for healthy cows and cows with mastitis, respectively

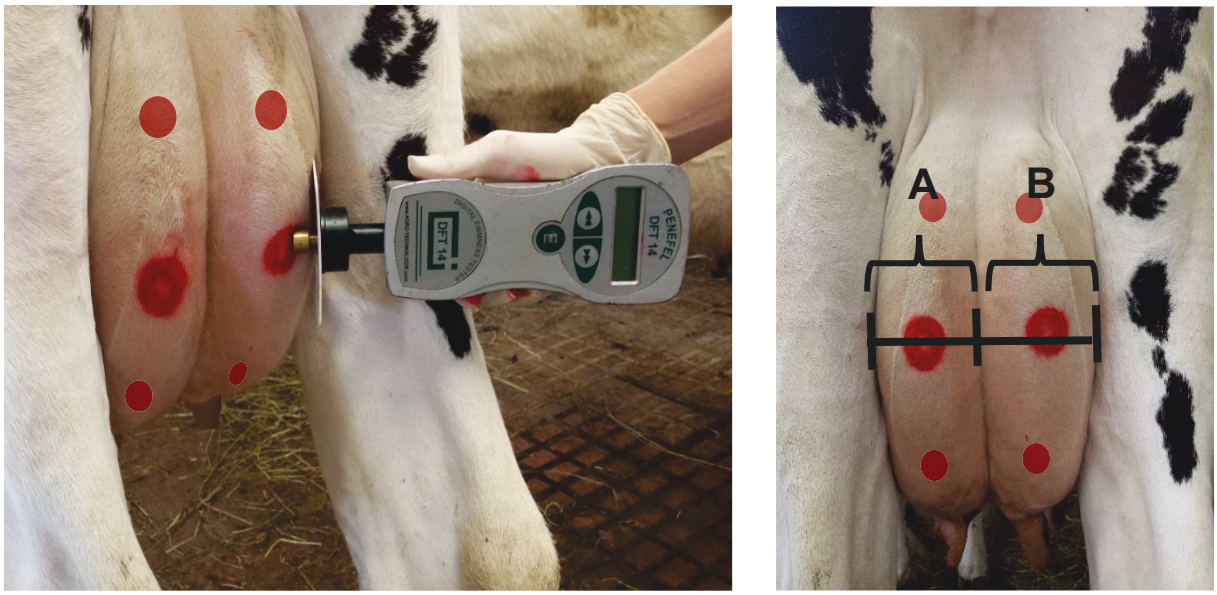

Figure 1. Measuring udder firmness using a dynamometer (Penefel DFT 14; Agro Technologies, Forges-les-Eaux, France). Measuring points were marked with livestock paint crayons (Raidex, Dettingen, Germany). Difference in udder firmness between both hind quarters (Δ firmness) was calculated by subtracting the firmness of the left (A) and the right (B) quarter measured after milking. Udder morphology was evaluated by measuring the width and length of both hind quarters. A symmetric udder was defined as having a difference in width and length between both hind quarters < 2 cm

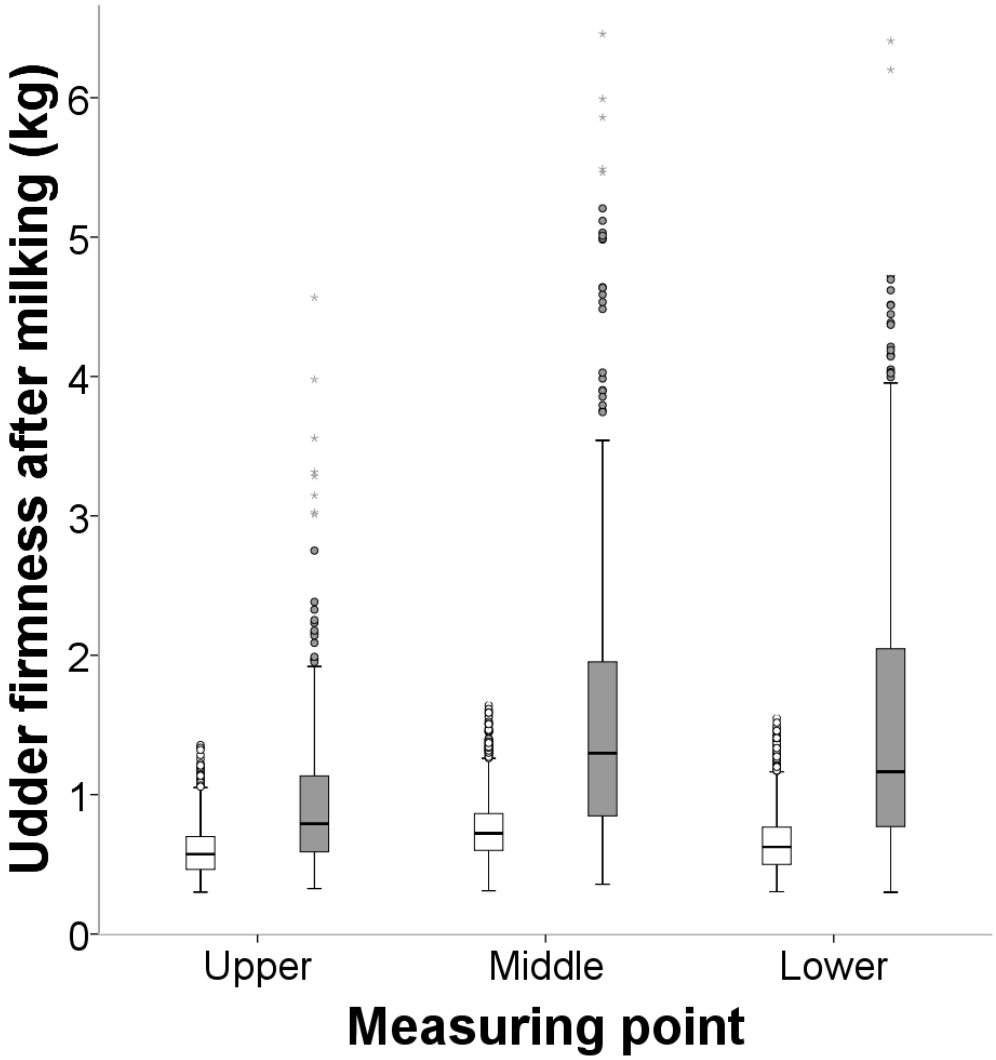

Figure 2. Udder firmness (kg) measured after milking on three locations (upper, middle, lower measuring point) within a given udder quarter in cows without (n = 95;☐) and with clinical mastitis (n = 45;■) using a dynamometer. Circles and asterisks indicate outliers and extreme outliers, respectively

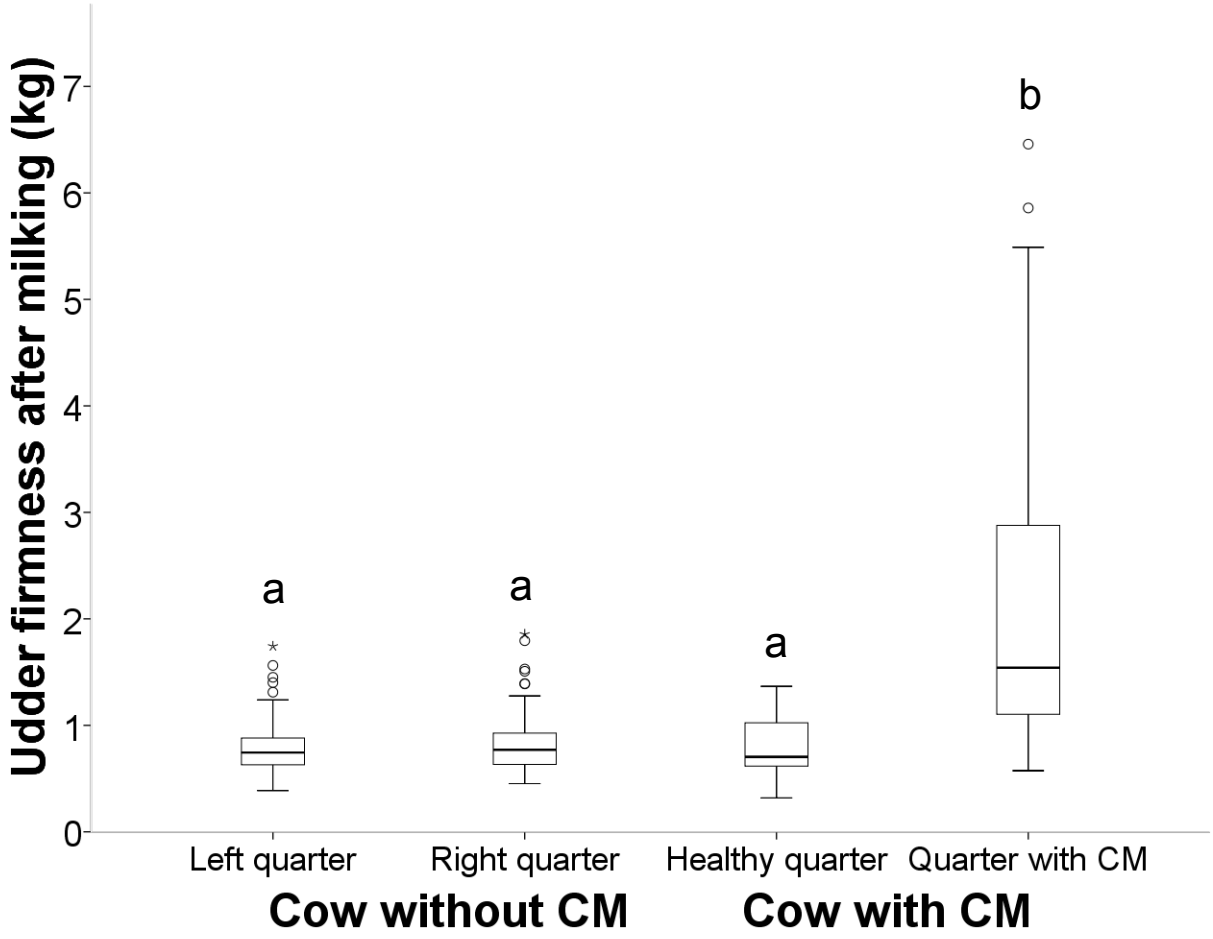

Figure 3. Udder firmness (kg) of hind quarters in cows without clinical mastitis (CM, n = 95) considering healthy and mastitic quarters in cows with CM (n = 45) using a dynamometer. Values with different lowercase letters differ (Kruskal-Wallis test; $P < 0.001$). Circles and asterisks indicate outliers and extreme outliers, respectively

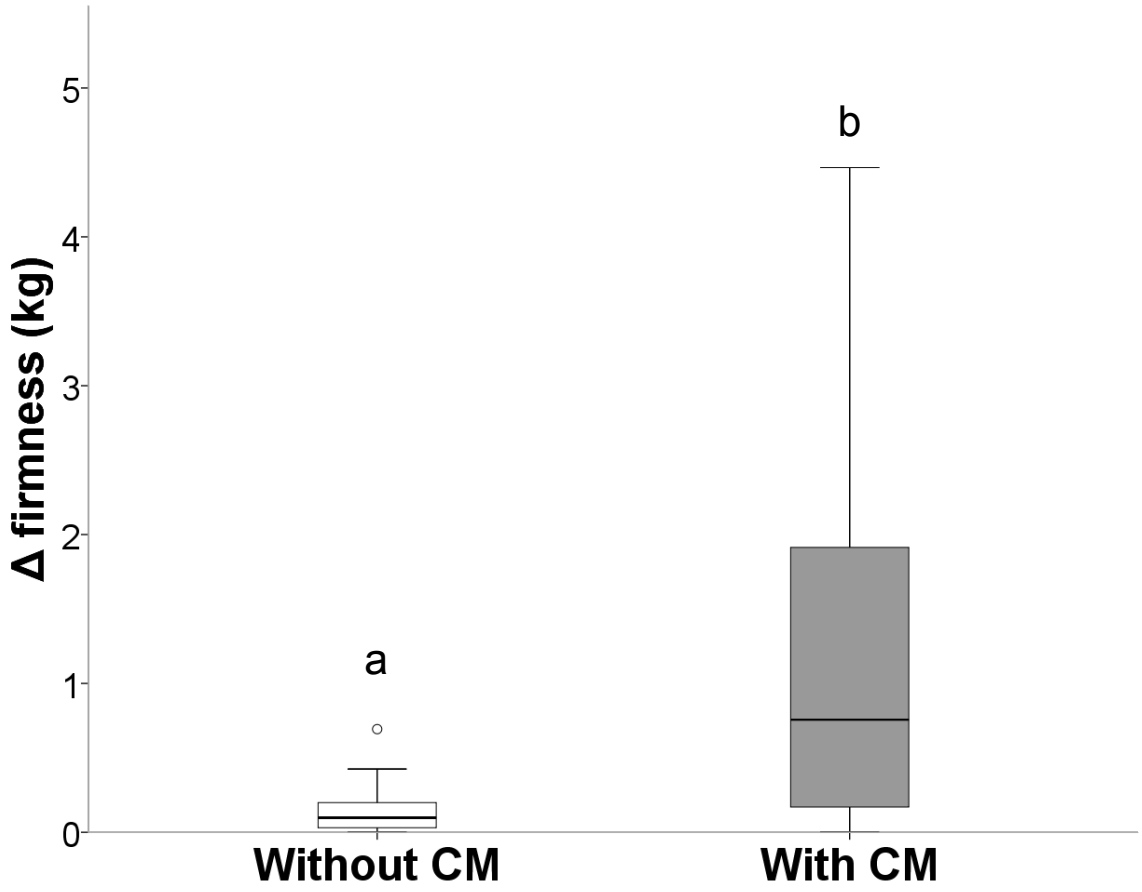

Figure 4. Difference in udder firmness between both hind quarters (Δ firmness, kg) in cows without (n = 95) and with clinical mastitis (CM; n = 45) measured with a dynamometer after milking. Values with different lowercase letters differ (Kruskal-Wallis test*; P* < 0.001). Circles indicate outliers

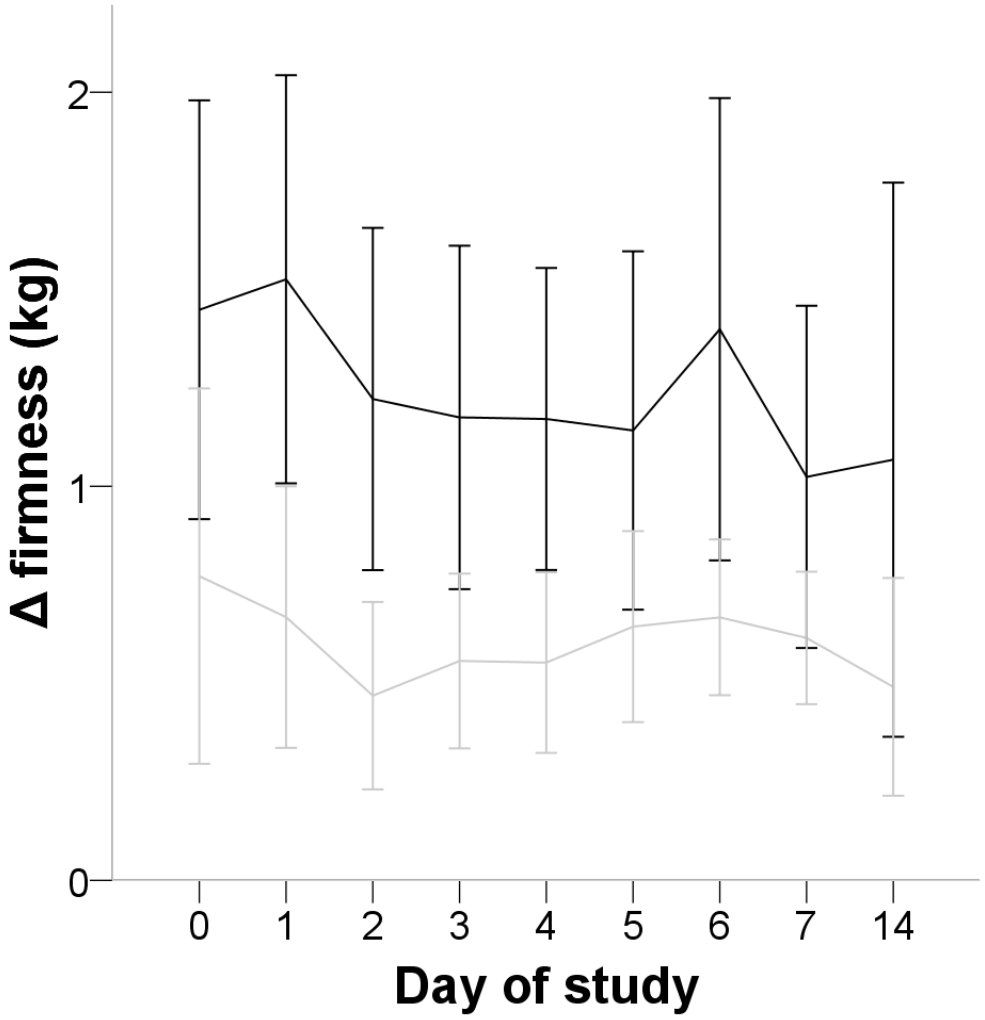

Figure 5. Difference of udder firmness between both hind quarters (Δ firmness, kg) in cows with mild to moderate (n = 24; light line) or severe clinical mastitis (n = 21; dark line) within 14 days after mastitis was diagnosed. Values of Δ firmness shown were generated by a linear mixed-model ANOVA

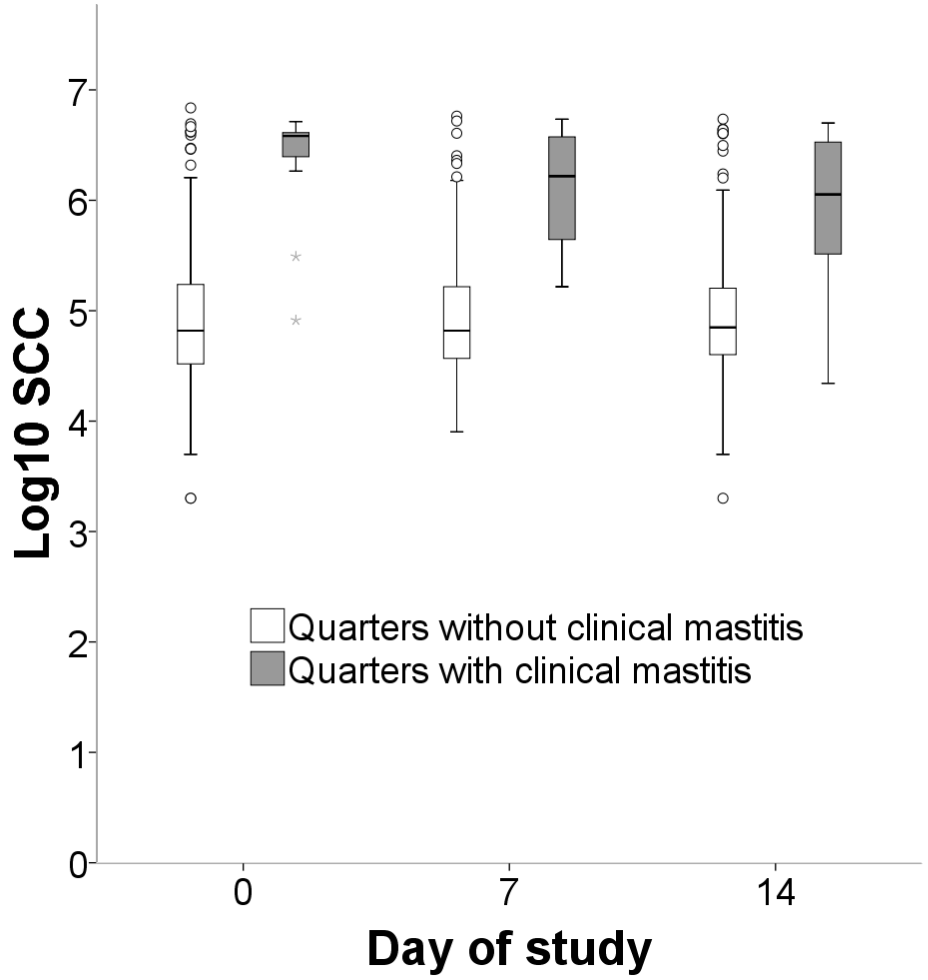

Figure 6. Boxplot of $\log_{10}$ SCC for each study day in quarters without (n = 235) and with clinical mastitis (n = 45). Circles and asterisks indicate outliers and extreme outliers, respectively

3.6 Discussion

The objective of our study was to evaluate if udder firmness can be used to detect CM. Some efforts have been undertaken to evaluate symptoms of mastitis others than abnormalities in the milk such as pain (Fitzpatrick et al., 2013) or radiated heat emitted by the udder skin (Hovinen et al., 2008). To our knowledge, only preliminary evidence (Rees et al., 2013) has been presented on udder firmness as a criterion to diagnose CM using a validated device. Studies using this device were carried out only in healthy cows (e.g., Bertulat et al. 2013) but not in cows with mastitic quarters.

Our results clearly indicate that an udder examination to diagnose a CM should be conducted after milking as udder firmness in quarters with CM (1.036 kg, 0.704 to 1.702 kg) was significantly higher than in quarters without CM (0.636 kg, 0.514 to 0.783 kg) after milking. This is in line with a previous recommendation (Radostits et al., 2006), describing that clinical examination of the mammary gland is of greatest value when the udder has been recently milked. We assume that the effect of milk filling the udder confounds the difference in firmness due to inflammation before milking.

In previous studies (Rees et al., 2013; Rees et al., 2014) and the current trial, median milking-induced decrease of udder firmness in healthy quarters was significant. It ranged from 0.763 kg (IQR: 0.426 to 1.570 kg; Rees et al., 2014) over 1.207 kg (IQR: 0.649 to 1.914 kg) in our study to 1.950 kg (IQR: 1.458 to 2.827 kg; Rees et al., 2013). A decrease in firmness can be explained by a drop in pressure due to milking.

Second, we tested the effect of measuring point within a quarter to identify the best diagnostic location. Firmness between the three locations within a given quarter was compared to determine a potential firmness gradient. Firmness measured before and after milking differed between the three locations ($P < 0.001$) both in cows without (n = 1,848 and 2,081) and with CM (n = 356 and 399). This finding is in agreement to a previous study (Bertulat et al., 2012). However, they found the lower measuring point to be the firmest, instead of the middle measuring point as in our study, and assumed Pascal's law (i.e., in fluids, pressure is highest at the lowest level) to be one explanation for this finding. In their study, however, only cows after dry-off were used. It was shown that only 38 to 47% of the total milk yield is stored within the cistern i.e., in the lower part of the udder (McKusick et al., 2002). When a cow is not milked or recently dried-off, the amount of milk stored in the cistern increases leading to a higher udder firmness in the lower part of the udder (Bertulat et al., 2012). We assume that firmness measured in the upper point primarily represents connective tissue of the suspensory apparatus and firmness of the lower measuring point is biased by the proximity to the gland cistern, which is a soft structure due to its cavernous character. Interestingly, our results

indicate that difference in udder firmness between the middle and lower measuring point within each quarter provides diagnostic value for the detection of inflammation as in quarters with CM there was no difference in firmness after milking between these two measuring points (P = 0.257). This could be explained by an udder edema as one clinical sign associated with CM (Fitzpatrick et al., 2013). This edema is likely to sink to the lowest part of the udder resulting in an increased firmness on the lower measuring point. Regardless of the reasons for firmness distribution, however, our data as well as previously published data (Bertulat et al., 2012) emphasize the importance of a defined measuring point for a repeatable measurement.

Firmness of front udder quarters was not studied for practical reasons. As cows stood side-by-side in the milking parlor, reaching the front quarters with the dynamometer was not possible. Additional studies are warranted to evaluate if udder firmness of front quarters could be used for CM diagnosis as well.

After evaluating firmness before and after milking and firmness gradients within a given quarter, we used firmness values assessed on the middle measuring point at D0 to evaluate differences in udder firmness between quarters without and with CM, as this location has been shown to have the greatest value for these calculations (Tucker et al., 2009; Bertulat et al., 2013). Firmness in quarters with CM was higher (P < 0.001) compared to quarters without CM. Firmness of healthy quarters did not differ when considered either as a dependent (within one healthy cow; P = 0.369) and or as an independent variable (throughout all enrolled cows without and with CM; P = 0.419). This demonstrates that the udder tissue of a given quarter is not affected by an inflammation of the neighboring quarter. Thus, an increase of udder firmness of the quarter with CM did not affect the firmness of its healthy neighboring quarter. Also, udder firmness of healthy quarters did not differ. Our data indicate that a healthy quarter next to a quarter with CM could be used as a negative control. Therefore, we calculated Δ firmness as a parameter on the cow level to reduce data clustering and provided a diagnostic approach by using the cow as her own negative control.

After milking, cows with CM had a higher Δ firmness compared to healthy cows (P < 0.001) due to one firmer udder quarter with CM. Further evaluation of factors potentially influencing udder firmness revealed that udder firmness of first-parity cows was only increased by CM. This could be explained by the smaller udder shape of young cows (Fossing et al., 2006) and that parenchymal and alveolar cells in the udder grow until fifth or sixth parity (Klaas et al., 2004). Furthermore, udders of first-parity cows are not yet influenced by CM in previous lactations. Udder firmness of older cows was affected by CM (P < 0.001), milk yield per day (P < 0.001) and DIM (P < 0.001). Our results might be explained by the different shape of lactation curves (Horan et al., 2005) and the decrease of secretory tissue during the declining phase of lactation (Klaas et al., 2004) resulting in higher variation of milk yield within one lactation in

older cows and in a softer udder in the course of one lactation, respectively. Interestingly, milk yield per day influenced udder firmness even after milking in cows in their second and greater parity. It has been described that udder tissue will be more active producing milk, especially at the beginning of the lactation as milk will be stored in ducts and cisterns, leading to a harder udder even when examined immediately after milking (Fossing et al., 2006).

We had expected gram-negative pathogens to cause firmer udders due to the well-known fact that the majority of cases with severe symptoms are caused by gram-negative pathogens (Oliveira et al., 2013). Furthermore, changes in echogenicity in cows with CM were more pronounced by some gram-negative pathogens (e.g., Fasulkov et al., 2012). Interestingly, we could not confirm those findings with our data set which might be due to the relative small number of CM caused by gram-negative pathogens (n = 10). In a previous study (Oliveira et al., 2013) milk samples of 34.1% of cows showing mild symptoms of CM were bacteriologically negative. This could further explain the non-existent influence of gram-staining characteristics in our study. Symmetry of hind quarters did not affect Δ firmness. Cows without and with CM, however, had symmetric hind quarters in 86.3% (82 out of 95 cows) and in only 15.6% (7 out of 45 cows), respectively ($P < 0.001$).

We set out to utilize udder firmness as a cow-side indicator for CM by establishing objective firmness thresholds for the differentiation between cows without and with CM. ROC curves were used to determine the diagnostic value of udder firmness measurements for detecting CM, which was defined by visible abnormalities in the milk according to the International Dairy Federation (IDF, 1999). The AUC of our study ranged from 0.722 (first-parity cows) to 0.817 (second- and greater-parity cows), indicating that Δ firmness provides a very accurate test (Swets, 1988; Ospina et al., 2010). A threshold of > 0.282 kg and > 0.425 kg Δ firmness provided the best combination of sensitivity and specificity i.e., identified 64.3% of first-parity cows and 62.5% of older cows as truly suffering from CM. The positive predictive value (i.e., the probability that CM was present when the test was positive) using these thresholds was higher for older cows (90.9%) compared to first-parity cows (69.2%). Sensitivity of the method is of particular importance in mastitis detection (Pyörälä, 2003) as improved sensitivity of a mastitis detection technique improves herd udder health (Kamphuis et al., 2010). On the other hand, the evaluation method should be highly specific for healthy quarters to avoid separating normal milk (Hovinen and Pyörälä, 2011) and potentially treating healthy cows with antibiotics. Our firmness thresholds provided either a high sensitivity or high specificity, but could not achieve both of the target values set by the ISO (2007). The trade-off between sensitivity and specificity has already been described in a systematic review (Rutten et al., 2013) for sensor studies addressing mastitis detection. None of the studies mentioned in this review met the ISO/FDIS 20966 limit of sensitivity and specificity (ISO, 2007).

Considering the range of the positive predictive value, the diagnostic value of udder firmness (69.2 to 93.1), however, was equivalent to that of milk thermography (78.8 to 100; Polat et al., 2010) and even better than e.g., electrical conductivity of milk (7 to 16; Hovinen and Pyörälä, 2011). However, to achieve a lower sensitivity of 50% (Hillerton et al., 2000), > 0.693 kg, > 0.387 kg and > 0.820 kg Δ firmness should be used as thresholds for all cows, first-parity cows and for older cows, respectively. A sensitivity of 77% (Pohl et al., 2013) is achieved by a Δ firmness threshold of > 0.153 kg, > 0.021 kg and > 0.167 kg in all cows, first-parity cows and for older cows, respectively. By using a Δ firmness threshold of > 0.167 kg, > 0.139 kg and > 0.178 kg, a specificity of 70% (Hillerton et al., 2000) is achieved for all cows, first-parity cows and for older cows, respectively. A threshold of > 0.149 kg, > 0.128 kg and > 0.167 kg Δ firmness achieves a specificity of minimum 66% (Pohl et al., 2013) in all cows, first-parity cows and for older cows, respectively.

Beside a device to assess changes in pain sensitivity in the course of a CM i.e., a pressure algometer (Fitzpatrick et al., 2013), the dynamometer would be the first measuring device evaluating a symptom of the gland itself to diagnose CM in addition to milk-based detection systems such as cell count or conductivity. Combining both approaches to detect CM would be closer to the definition of this disease by the IDF (1999) characterizing a CM as visible abnormalities in the milk and/or in the udder. Also, combining several indicators for CM could improve detection of CM (Hovinen and Pyörälä, 2011).

We followed the cows for 14 days after the initial diagnosis of CM. Surprisingly, Δ firmness of cows with CM was higher compared to healthy cows even 14 days after CM was diagnosed. Hence, infected quarters were firmer until the end of the observation period indicating a healing process of over two weeks. Day of study had no effect on udder firmness. These results are consistent with recently published data suggesting that the recovery period after a CM expressed as reduced milk yield, elevated lactate dehydrogenase activity, lower milking frequency, and elevated inter-quarter yield ratio continued for weeks after the antibiotic treatment (Fogsgaard et al., 2015). As clinical cure was defined as the presence of a normal gland and normal milk (Schukken et al., 2013), there was no clinical cure regarding this definition likewise. Twenty-four and 30 out of 45 quarters with CM showed a normal milk on D7 and D14, respectively but were considerably firmer than healthy quarters. This discrepancy could be explained by the reaction of the connective udder tissue towards sustained immune response such as release of chemokines (Wellnitz and Bruckmaier, 2012) leading to a firmer udder even after return to a normal appearance of milk. Furthermore, a sensitivity of 80% for visual inspection of milk for CM was reported (Hillerton, 2000). Hence, cows with minimum abnormalities of the milk could have been judged as healthy by the milking personnel (n = 9). A bacteriological cure on D14 was observed in 22 out of 45 quarters with CM. The relationship

between bacteriological cure and clinical cure was moderate. In bacteriologically cured cases, 73% (16 out of 22 cows) also showed clinical cure, whereas the bacteriologically non-cured cases showed 54% clinical cure. Similar results were reported by Schukken et al. (2013). The variance in severity and the variety of pathogens involved could be an explanation as different pathogens lead to different cure rates (Schukken et al., 2013). As cows with CM were considered as healthy cows in the statistical model as soon as they were cured, they potentially could have biased the difference in udder firmness between cows without and with CM throughout the 14 days. Therefore, we removed cows with CM, which had normal milk, and recalculated the mixed models. This approach, however, did not change the results indicating the absence of a bias.

As on D0, parity affected udder firmness on all study days (P = 0.016). Regardless of CM, older cows had a higher (P = 0.023) Δ firmness compared to first-parity cows on all study days. As it was mentioned before, first-parity cows have not fully developed and a more uniform udder tissue compared to older cows. Moreover, firmness is not biased by previous cases of CM. Severity of CM influenced udder firmness throughout the 14 days as cows with mild to moderate CM showed lower Δ firmness compared to cows with severe CM. This indicates that cows with systemically signs of a CM also suffer from a firmer udder.

3.6.1 Clinical and bacteriological cure

While clinical cure rates were similar in cows suffering from mild to moderate CM and severe CM, only 25% (6 out of 24) of cows with severe CM compared to 52% (11 out of 21) of cows with mild to moderate CM were bacteriologically cured even though the distribution of bacteriologically negative, gram-positive, and gram-negative samples were similar in cases of mild to moderate and severe CM. This finding is similar to previous observations that the probability of bacteriological cure decreases when the cow is systemically ill (Steeneveld et al., 2011).

3.6.2 Study limitations

In all studies investigating udder health and mastitis several biases might have influenced the results (Dufour et al., 2012). First, the investigator in our study could not be blinded to the udder health status of the cow due to logistical limitations. We considered it unlikely that the non-blinded investigator affected the outcome of the study because measurements were conducted with a digital device (dynamometer) and a standard operation procedure (Bertulat et al., 2012) was implemented. As only a limited number of CM cases (n

= 45) from one herd was included, study population may not represent all variations and clinical signs of CM (Hovinen and Pyörälä, 2011).

It would be interesting to determine to what extent udder firmness measured by a dynamometer could improve in-line detection of CM in automatic milking systems. Further research is warranted to evaluate potential confounders such as incomplete emptying of the udder after milking (Hovinen and Pyörälä, 2011). Also, it needs to be studied if the firmness of front quarters could serve as a diagnostic tool likewise. In addition, a more practical approach for diagnosing CM such as palpation could be investigated in further studies. Especially because a palpation scoring system was demonstrated to have good within-observer repeatability (Rees et al., 2014). It remains unclear if udder firmness can be used as a predictor for the prognosis of a CM or the cure of inflammation.

3.7 Conclusions

Assessing udder firmness with a dynamometer is a useful tool to distinguish between healthy and inflamed quarters. To diagnose CM, udder of cows should be examined after milking in the middle or lower part of a given udder quarter. Additionally, different thresholds for first-parity and older cows should be used to reduce bias by parity. The results of our study indicate that cows with CM need a period longer than two weeks to recover.

3.8 Acknowledgements

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4 PUBLICATION II

Effect of heat stress on concentrations of fecal cortisol metabolites in dairy cows

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4.1 Contents

The negative impact of heat stress on health and productivity of dairy cows is well known. Heat stress can be quantified with the temperature-humidity-index (THI) and is defined as a THI $\geq$ 72. Additionally, animal welfare is affected in cows living under heat stress conditions. Finding a way to quantify heat stress in dairy cows has been of increasing interest over the past decades. Therefore, the objective of this study was to evaluate concentrations of fecal glucocorticoid metabolites [i.e., 11,17-dioxoandrostanes (11,17-DOA)] as an indirect stress parameter in dairy cows without heat stress (DOA 0), with heat stress on a single day (acute heat stress; DOA 1) or with more than a single day of heat stress (chronic heat stress; DOA 2). Cows were housed in five farms under moderate European climates. Two statistical approaches (approach 1 and 2) were assessed. Using approach 1, concentrations of fecal 11,17-DOA were compared among DOA 0, DOA 1 and DOA 2 samples regardless of their origin (i.e., cow; unpaired comparison with a one-way ANOVA). Using approach 2, a cow was considered as its own control i.e., 11,17-DOA were treated as a cow-specific factor and only paired samples were included in the analysis for this approach (paired comparison with *t*-tests). In approach 1 ($P = 0.006$) and approach 2 ($P = 0.038$) 11,17-DOA values of cows under acute heat stress were higher compared to those of cows without heat stress. Our results also indicate that acute heat stress has to be considered as a confounder in studies measuring fecal glucocorticoid metabolites in cows to evaluate other stressful situations.

4.2 Introduction

The negative impact of hot and humid climate conditions on animal health, productivity, and reproductive performance of dairy cows is well known (West et al., 2003; Collier et al., 2006). Heat stress can be quantified with the temperature-humidity-index (THI) and is defined as a THI $\geq$ 72 (Ravagnolo et al., 2000). Increasing milk yield per cow over the last decades (Hansen, 2000) has resulted in increased metabolic heat production (Kadzere et al., 2002). In particular high-yielding dairy cows became less tolerant to hot climate conditions (West, 2003). Cows exposed to heat stress show reduced conception rate (De Rensis et al., 2002; Kadzere et al., 2002; Schüller et al., 2014). Additionally, milk yield declines in cows with heat stress (Ravagnolo et al., 2000; West, 2003). Therefore, heat stress in dairy cows is an important factor negatively affecting profitability (St-Pierre et al., 2003; Collier et al., 2006).

Whereas the influence of heat stress on animal welfare of extensively managed cattle has been reviewed (Silanikove, 2000), there is a lack of information on the impact of heat stress on animal welfare in confinement housing. Animal welfare has been defined as the physical and psychological well-being of animals (Hewson, 2003). Animal welfare issues are

of increasing importance both in research and to the public (Rushen et al., 2007; von Keyserlingk et al., 2009, 2013). Therefore, measuring stress or stress responses in farm animals has become the subject of recent research efforts in order to objectively evaluate animal welfare (de Vries et al., 2013; Endres et al., 2014). Measurement of glucocorticoid metabolites [i.e., 11,17-dioxoandrostanes (11,17-DOA)] in fecal samples is a scientifically established and practical method to determine chronic stress in cows (Palme and Möstl, 1997; Palme et al., 1999; Morrow et al., 2002; Möstl and Palme, 2002). A time lag of 8 to 16 h between stress exposure and elevated fecal 11,17-DOA concentrations has been demonstrated (Palme, 2005; Morrow et al., 2002; Palme et al., 2000). Heat-induced suppression of metabolic processes has been shown to cause a decline in the concentrations of somatotropin (McGuire et al., 1991) as well as triiodothyronine and thyroxin (Johnson et al., 1988). Elevated plasma cortisol concentrations were found in three cows after 12-h exposure to heat stress in an environmental chamber (13.0 ng/mL; Christison and Johnson, 1972) and in 16 cows after seven days when housed in a dry lot without access to shade or other cooling facilities (3.3 ng/mL; Elvinger et al., 1992). Furthermore, Muller et al. (1994) studied 20 cows kept in lots with differently designed shade structures and found higher concentrations of plasma cortisol (3.3 nmol/L) in not-shaded compared to shaded cows. Sampling feces instead of blood has the advantage of an easy collection and stress-free handling of the cow (Möstl and Palme, 2002). Concentrations of fecal glucocorticoid metabolites, blood cortisol, and adrenal activity are directly related (Palme et al., 1999; Morrow et al., 2002). These findings lead to the hypothesis that fecal cortisol can be an indicator for heat stress in cows.

The overall objective of this study was to evaluate if acute and chronic heat stress in individual dairy cows is associated with concentrations of fecal 11,17-DOA. Specifically, the study set out to determine the relationship of acute and chronic heat stress and 11,17-DOA concentrations in fecal samples of late-lactating dairy cows housed in moderate European climates considering the effect of farm.

4.3 Materials and methods

4.3.1 Animals and housing

The study was conducted on five commercial dairy farms in Germany (farms 1 and 2), Hungary (farms 3 and 4) and France (farm 5) between June and September 2012. In total, 115 predominantly Holstein-Friesian cows (50 primiparous, 65 multiparous) were included in the study. Cows on all farms were continuously housed indoor. A summary of the farms is given in table 1. On the day of enrollment, cows were 334 $\pm$ 74 DIM (mean $\pm$ SD) and had an average

milk yield of 9,771 ± 2,580 kg per 305 days. However, this was at the end of lactation when supposedly their milk production was low.

4.3.2 Experimental design

As soon as the climate loggers were secured and recording of climate data started, every farm was regularly visited at a consistent time (e.g., farm 1: 0805 ± 1.4 h; mean time ± SD) once a week at the minimum. All cows supposed to get dried-off seven days after a given visiting day were enrolled in the study and that day was marked as the day of their enrollment. On the day of enrollment, the overall status of all cows was checked by recording breathing and heart rate, rectal temperature and rumination. Lameness was assessed according to the 5-point lameness score developed by Sprecher et al. (1997). To measure fecal 11,17-DOA concentrations, fresh fecal samples were collected. Sampling procedures for fecal glucocorticoid metabolites were described previously (Bertulat et al., 2013). On the visit seven days later i.e., the day of dry-off, enrolled cows underwent the same procedure as on the day of enrollment. Additionally, average milk production of the last four days before dry-off was retrospectively determined using on-farm herd management software programs. To ensure an equal level of performance, only cows with an average milk production of > 16 kg per day in the last four days before dry-off were included in the study.

Cows with an impaired overall status and a lameness score ≥ 3 on the day of enrollment or the day of dry-off were not enrolled or finally included, respectively. Farmers were committed to report any events or activities interfering with data collection. Claw trimming or treatments interfering with the milk production (e.g., diuretic products) were not performed during the study.

4.3.3 Fecal glucocorticoid metabolites

Extraction and measurement of 11,17-DOA were performed utilizing an 11-oxo-etiocholanolone enzyme immunoassay as described previously (Palme and Möstl, 1997). All samples were analyzed in duplicate. Intra-assay and inter-assay coefficients of variation were calculated. Concentrations of 11,17-DOA are stated in nanograms per gram of wet feces. Caused by the time lag between elevated stress levels and increased 11,17-DOA concentrations, 11,17-DOA concentrations determined for a given day were considered to represent the stress level of the previous day. This was possible, because every farm was visited at a consistent time as mentioned earlier. According to previous studies (Christison and Johnson, 1972; Alvarez and Johnson, 1973), acute and chronic heat stress was defined as a

single day or more than a single day of heat stress, respectively. Cows exposed to heat stress on the sampling day were not included in any of the calculations.

4.3.4 Statistical approaches

Two statistical approaches were used to compare concentrations of 11,17-DOA in fecal samples between days without heat stress (DOA 0) and with acute (DOA 1) or chronic heat stress (DOA 2). Using approach 1, concentrations of fecal 11,17-DOA were compared among DOA 0 (n = 116), DOA 1 (n = 25) and DOA 2 (n = 27) samples regardless of their origin (i.e., cow). Because the influence of individual variations in fecal 11,17-DOA concentrations can be reduced if an animal is considered as its own control (Palme et al., 2000), a second analysis was performed (i.e., approach 2). Considering cow-specific concentrations of 11,17-DOA as the outcome variable, the cow was the unit of interest, with two repeated measures (Koch et al., 1980), i.e., one fecal sample on a day without heat stress (DOA 0) compared to another day without heat stress (DOA 0.1), a single day of heat stress (DOA 1) or more than a single day of heat stress (DOA 2), treated as paired samples, respectively. Therefore, using approach 2, concentrations of 11,17-DOA were treated as a cow-specific factor and only samples of each of 21 (DOA 0 and DOA 0.1), 22 (DOA 0 and 1) and 8 (DOA 0 and 2) cows were included in the analysis for this approach. On the day of enrollment, these cows were 333 $\pm$ 53 DIM and had an average milk yield of 9,737 $\pm$ 2,186 kg per 305 days. Average daily milk production for the four days before dry-off was 20.5 $\pm$ 2.9 kg. In this study, chronic heat stress occurred for a period of 8 $\pm$ 6 days in approach 1 and 3 $\pm$ 2 days in approach 2.

4.3.5 Climate data

Climate loggers (Tinytag Plus II Data Loggers; Gemini Loggers Ltd., Chichester, UK) were installed in every barn to record local climate data as it was proven that climate data are confounded when recorded in the vicinity of farms (Schüller et al., 2013). They were secured at a beam 3 m from the ground in the center of the late-lactation pen of each farm. Maximum length of study pens was 150 m (farm 5). Therefore, maximum distance from loggers to the cows was approximately 75 m. Loggers hourly measured ambient temperature (AT) from −25 to 85 °C with an accuracy of $\pm$ 0.3 °C and a resolution of 0.01 °C and relative humidity (RH) from 0 to 100% with an accuracy of $\pm$ 3% and a resolution of 0.3%. They were calibrated by the manufacturer at the beginning and the end of the study and accuracy was checked. The measured data were downloaded after completion of the study. The THI was calculated using the formula reported by Kendall and Webster (2009): THI = (1.8 x AT + 32) − [(0.55 − 0.0055 x RH) x (1.8 x AT − 26)]. Hourly values were averaged for each study day individually. Days of

heat stress were defined as days with a mean THI $\geq$ 72 (Armstrong, 1994; Ravagnolo et al., 2000). Precise data regarding the period with climate recorded is provided in table 1.

4.3.6 Statistical analysis

Data were entered into Excel (Office 2010; Microsoft Deutschland GmbH, Munich, Germany) and Access (Office 2010; Microsoft Deutschland GmbH, Munich, Germany) and statistical analysis were performed with IBM SPSS Statistics for Windows software (version 20.0; IBM Deutschland GmbH, Ehningen, Germany). The normal distribution of the climate data and 11,17-DOA values was tested by calculating a quantile-quantile (Q-Q) plot and performing a Shapiro-Wilk test. Data were not normally distributed ($P = 0.017$). Therefore, data were $\log_{10}$ transformed before analysis to achieve normal distribution; but back-transformed values are reported. The differences between 11,17-DOA values in cows with no (DOA 0; n = 116), acute (DOA 1; n = 25) or chronic (DOA 2; n = 27) heat stress were determined with a one-way ANOVA considering the farm as fixed factor. Mean differences were determined with the least significant difference post hoc test. The effect of acute (n = 22) or chronic (n = 8) heat stress on concentrations of fecal 11,17-DOA was statistically determined by a *t*-test for paired samples using approach 2. Additionally, a *t*-test for paired samples was performed to assess if 11,17-DOA concentrations in cows without heat stress (n = 21) differed between both samples. In detail, concentrations of 11,17-DOA in the two given fecal samples were compared in cows without heat stress (n = 21) and with heat stress on a single day (n = 22) or with more than a single day of heat stress (n = 8) prior to feces sampling, respectively. For better assessment of the variations in 11,17-DOA concentrations using approach 2, the changes in 11,17-DOA concentrations in cows not, acutely or chronically heat stressed were calculated relative to the individual baselines i.e., 11,17-DOA on a day without heat stress (11,17-DOA_{rel}). These were computed for each individual cow using a formula described by Bertulat et al. (2013). The effect of no, acute and chronic heat stress on 11,17-DOA_{rel} values in individual cows was evaluated in a linear mixed-model ANOVA.

Minimum, maximum, median and interquartile range of 11,17-DOA values on day DOA 0, DOA 0.1, DOA 1 and DOA 2 were calculated for both statistical approaches.

All THI data were calculated for the periods with climate recorded both for all five farms and for the four farms with days of heat stress. Differences between mean daily THI of the farms were analyzed using a Kruskal-Wallis one-way analysis. Number of days of heat stress was compared and statistical significance was estimated by using a Chi-squared test.

4.4 Results

A total of 230 fecal samples were collected and climate data of 297 days were analyzed.

4.4.1 Climate data

Descriptive statistics for the THI are shown in table 1. Number of days with heat stress ranged from 0% (farm 5) to 29.4% (farm 3). Number of days with heat stress differed among all farms ($P = 0.05$), but did not differ among farms 1 to 4 ($P = 0.768$). Mean daily THI tended to differ among all farms ($P = 0.149$; table 1). Mean daily THI did not differ ($P = 0.563$) among the four farms with days of heat stress.

4.4.2 Heat stress and fecal glucocorticoid metabolites

The intra- and inter-assay coefficients of variation for the 11-oxo-etiocholanolone enzyme immunoassay were 10.0 and 13.9%, respectively. Minimum, maximum, median and interquartile range of 11,17-DOA concentrations in cows with and without heat stress using both statistical approaches are summarized in table 2. Data used for approach 1 and farms 1 to 4 are plotted in figure 1.

Descriptive statistics for 11,17-DOA values are shown in table 2. Using approach 1, the effect of no (DOA 0; n = 116), acute (DOA 1; n = 25) or chronic (DOA 2; n = 27) heat stress as well as the farm as fixed factors on 11,17-DOA values was $P = 0.022$ and $P < 0.001$, respectively (figure 1). Cows without heat stress (DOA 0) had lower concentrations of 11,17-DOA compared to cows under acute heat stress (DOA 1; $P = 0.006$). Concentrations of 11,17-DOA did not differ between cows without heat stress and cows under chronic heat stress (DOA 2; $P = 0.840$). Hence, concentrations of 11,17-DOA were higher under acute heat stress and lower under chronic heat stress. Using approach 2, concentrations of 11,17-DOA in cows without heat stress (DOA 0 and DOA 0.1) did not differ ($P = 0.535$). Concentrations of fecal 11,17-DOA increased ($P = 0.038$; figure 2) in cows under acute heat stress compared to cows without heat stress. Concentrations of 11,17-DOA tended to decrease ($P = 0.15$; figure 2) in cows under chronic heat stress compared to cows without heat stress. In general, heat stress had an effect on changes of 11,17-DOA concentrations (11,17-DOA_{rel} values; $P = 0.014$). Compared to 11,17-DOA_{rel} values in cows without heat stress, 11,17-DOA_{rel} values in cows under acute heat stress were higher ($P = 0.026$) or did not differ ($P = 0.325$) in cows under chronic or acute heat stress, respectively.

Fecal 11,17-DOA concentrations differed among farms ($P < 0.001$) when using both statistical approaches.

Table 1. Housing information for the five farms investigated, number of cows included in the study and data regarding recorded temperature-humidity-index (THI) for the same farms. Heat stress was defined as days with a mean daily THI $\geq$ 72

Parameter	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Geographical position	52°15'N 13°22'E	51°58′ N 11°28′ E	47°10'N 19°47'E	46°33'N 20°40'E	50°14'N 2°30'E
Number of milking cows	851	1,149	989	620	800
Number of cows per pen	150	45	160	80	150
Pen size (m x m)	12 x 74	12 x 25	12 x 80	10 x 70	15 x 150
Area per cow (m^2)	5.9	6.6	6.0	8.8	15.0
Diet	roughage mix and concentrate via automatic feeder	TMR	TMR	TMR	TMR
Housing	free stall + cubicles	free stall + cubicles	free stall + cubicles	free stall	free stall
Flooring	concrete without slats	concrete without slats	concrete without slats	deep straw	deep straw
Cow 305-days milk production (kg)	10,500	10,130	9,669	8,800	8,600
Number of cows					
Approach 1	42	58	30	35	40
Approach 2	6	11	8	5	0

Continued

Table 1 (Continued). Housing information for the five farms investigated, number of cows included in the study and data regarding recorded temperature-humidity-index (THI) for the same farms. Heat stress was defined as days with a mean daily THI $\geq$ 72

Parameter	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Period with climate recorded	12th June to 12th September	22th June to 1st September	26th June to 15th August	9th July to 21th August	14th June to 20th July
Consecutive days (n)	93	72	51	51	37
Days of heat stress[1] (%)	20 (21.5)	18 (25.0)	15 (29.4)	13 (25.5)	0
THI (daily average)					
Median[2]	68.9	69.0	70.0	73.7	61.4
Interquartile range	66.7 to 71.9	65.0 to 71.3	66.1 to 72.6	70.5 to 76.3	58.8 to 63.3
Minimum	62.9	61.6	63.7	59.1	56.0
Maximum	78.6	76.8	76.6	78.1	69.9

[1]Values of a Chi-squared test revealed significant differences and no differences in the number of days of heat stress between all farms ($P = 0.05$) and for farms 1 to 4 ($P = 0.768$), respectively

[2]Values of a Kruskal-Wallis one-way analysis show a tendency to differ and no differences in median THI measured in the period with climate recorded between all farms ($P = 0.149$) and between farms 1 to 4 ($P = 0.563$), respectively

Table 2. Descriptive statistics of fecal concentration of 11,17-dioxoandrostanes (11,17-DOA, ng/g) in cows without heat stress (DOA 0), with heat stress on a single day (acute heat stress; DOA 1) or with more than a single day of heat stress (chronic heat stress; DOA 2) using two statistical approaches (approach 1 and 2). Using approach 1, fecal concentration of 11,17-DOA was compared among DOA 0 (n = 116), DOA 1 (n = 25) and DOA 2 (n = 27) samples regardless of their origin (i.e., cow). Using approach 2, a cow was considered as its own control i.e., concentration of 11,17-DOA was treated as a cow-specific factor and only samples of each of 22 (DOA 0 and 1) and 8 (DOA 0 and 2) cows were included in the analysis for this approach

	Concentration of 11,17-DOA (ng/g)		
Parameter	DOA 0	DOA 1	DOA 2
Approach 1 (unpaired comparison)			
Minimum	2.4	2.9	4.2
Maximum	85.2	61.1	61.7
Median	13.0	22.4	13.4
Interquartile range	8.0 to 21.3	11.9 to 38.8	6.6 to 18.0
Approach 2 (paired comparison between cows under acute heat stress)			
Minimum	3.7	2.9	-
Maximum	37.8	54.2	-
Median[1]	8.0	20.4	-
Interquartile range	5.0 to 12.3	11.8 to 38.3	-
Approach 2 (paired comparison between cows under chronic heat stress)			
Minimum	2.8	-	4.9
Maximum	43.8	-	23.1
Median[2]	17.8	-	15.3
Interquartile range	12.1.0 to 29.3	-	6.4 to 20.2

[1] *t*-test for paired samples: $P = 0.038$

[2] *t*-test for paired samples: $P = 0.15$

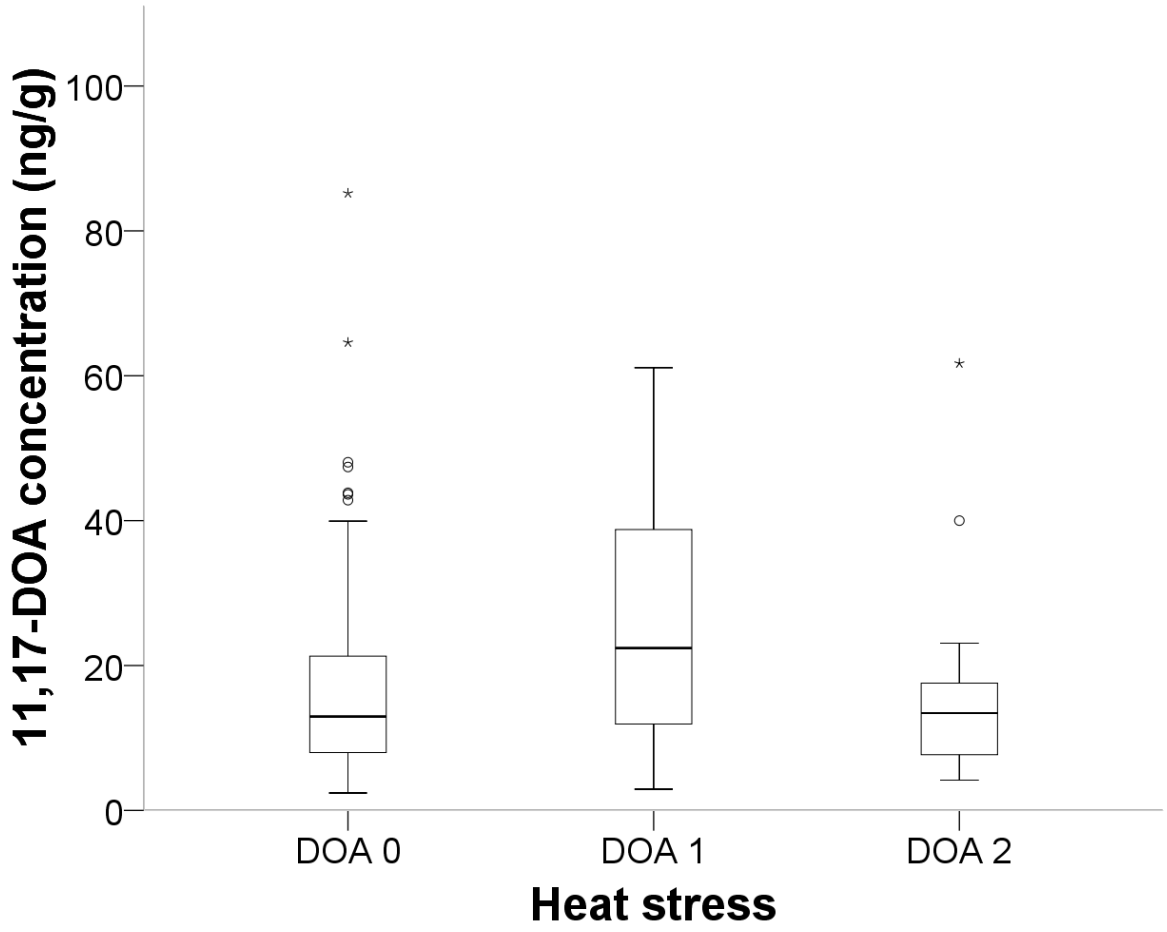

Figure 1. Boxplot of concentrations of fecal 11,17-DOA (ng/g) in cows without heat stress days (DOA 0; n = 116), with heat stress on a single day (DOA 1; n = 25) and with more than a single day of heat stress (DOA 2; n = 27) prior to feces sampling in approach 1, respectively

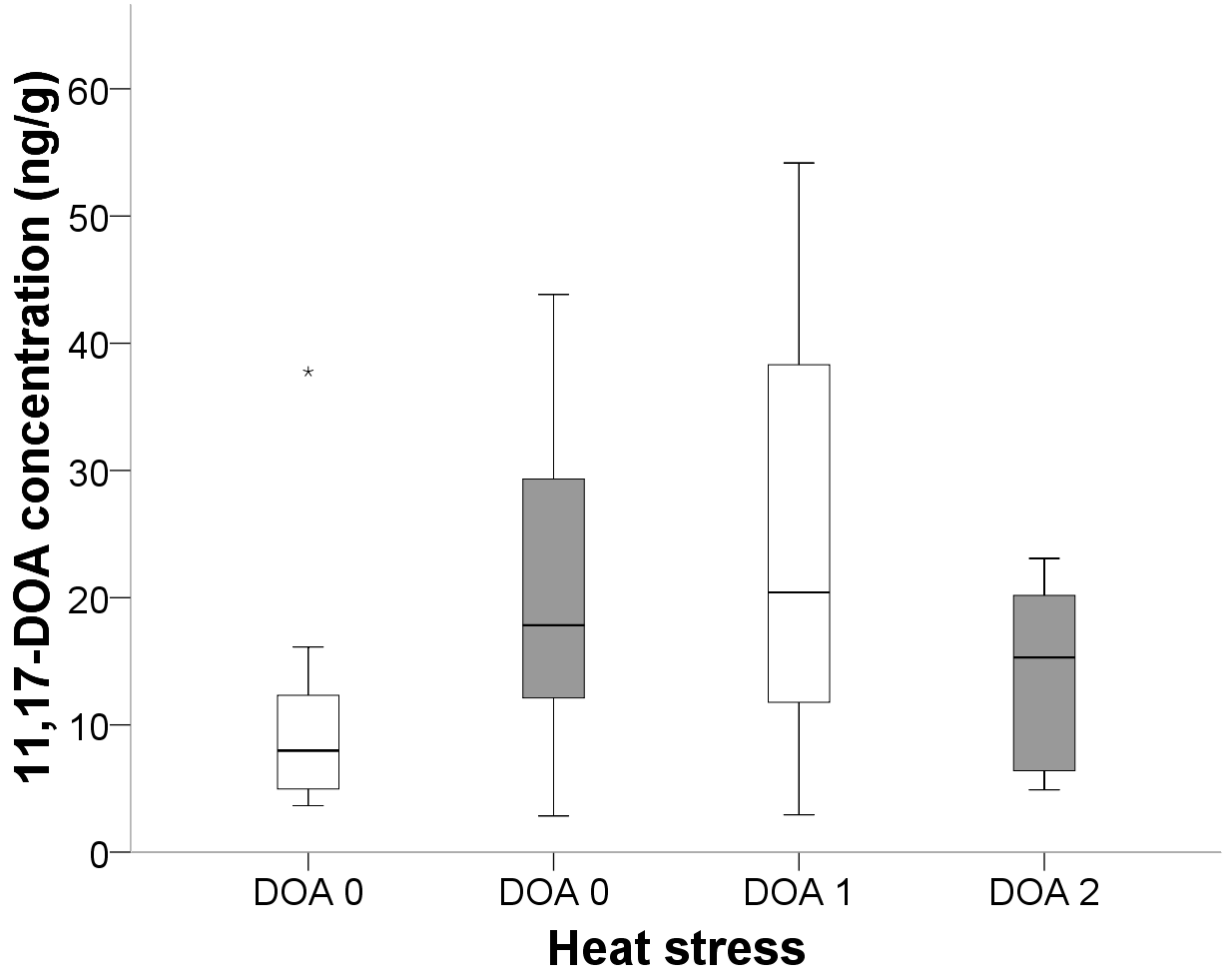

Figure 2. Boxplot of concentrations of fecal 11,17-DOA (ng/g) in cows without heat stress days and with heat stress on a single day (n = 22;☐) or with more than a single day of heat stress (n = 8;▩) prior to feces sampling with every cow as her own negative control in approach 2, respectively

4.5 Discussion

To our knowledge, this is the first study evaluating fecal 11,17-DOA concentrations as an indicator for heat stress in dairy cows. An increasing interest of public and research in the welfare of farm animals (Rushen et al., 2007; von Keyserlingk et al., 2009, 2013) results in an increasing need for a practical and objective evaluation of stress or stress responses in dairy cows. In this study, fecal 11,17-DOA were used to assess one possible stress response i.e., adrenocortical activity of the cortisol metabolism of dairy cows exposed to a high THI. Concentrations of fecal 11,17-DOA were higher under acute and lower under chronic heat stress using both statistical approaches. Also, cows under acute heat stress showed higher 11,17-DOA_{rel} values compared to cows without heat stress and cows under chronic heat stress. These results confirm findings of previous studies for glucocorticoids (Christison and Johnson, 1972; Alvarez and Johnson, 1973; Silanikove, 2000). The decline in hormone concentration under heat stress could be explained by a general attempt to reduce metabolic heat production via reduced concentration of key metabolic hormones (West, 2003; Bernabucci et al., 2010).

It was shown that fecal 11,17-DOA concentrations in dairy cows were influenced by factors such as transportation (Palme et al., 2000), handling (Pesenhofer et al., 2006) and milk yield (Bertulat et al., 2013). Furthermore, lameness as a painful process was hypothesized to result in stress (Barkema et al., 1994; Melendez et al., 2003). Additionally, high metabolic activity of high-yielding dairy cows has been considered as an influencing factor for elevated steroid metabolism (Wiltbank et al., 2006) and blood concentration of cortisol (Sartin et al., 1988). In this study, only healthy cows with similar milk production and stage of lactation were included to minimize variation. This was ensured by calculation of average milk yield in the last four days before dry-off and by conducting general clinical examination and lameness scoring resulting in homogeneity of study animals regarding milk production and health status. Also, stress-inducing claw trimming and drug applications were banned during the study period. To avoid confounding of data due to health problems and management decisions such as group changes as potential stressors within the transition period (Ingvartsen, 2006; Huzzey et al., 2011), we exclusively used late-lactating cows seven days prior to dry-off. As shown by Huzzey et al. (2011), concentrations of fecal cortisol metabolites three weeks before calving is not associated with the occurrence of postpartum diseases. Therefore, potentially biasing factors for fecal 11,17-DOA concentrations were considered to be negligible in this study.

Climate loggers used in this study were identical to loggers used in a previous trial (Schüller et al., 2013). They also installed climate loggers in every barn and found similar monthly mean THI in June (68.7), July (70.8), August (70.6) and September (65.7) of 2010 and 2011 to the THI values found in 2012 in this study. Mean THI instead of maximum THI was used in this study, because it was shown that mean THI is the most sensitive heat load

index for cows housed in the moderate climates with respect to fertility (Schüller et al., 2014) and that effects of THI on conception rates are more likely to be mediated through prolonged periods of time by a mean THI than by a high -but relatively short- daily maximum THI (Morton et al., 2007). There is a reduction in dry matter intake in heat stressed dairy cows, which prolongs the period of negative energy balance. This indirectly decreases first service conception rate due to alteration of normal folliculogenesis (De Rensis and Scaramuzzi, 2003).

In this study, cows of farm 5 did not experience heat stress. One could speculated that this was due to the largest area per cow i.e., the lowest stocking rate (Schüller et al., 2013) among all farms. Our study confirms previous observations (Silanikove, 2000; Hammami et al., 2013; Van laer et al., 2014) that heat stress also affected cows housed in temperate latitudes. Variation in climate as occurring in this study might challenge cows more intensively compared to continuously hot conditions due to a lacking adaptation to permanent hot climate (Kadzere et al., 2002). The maximum value of hourly assessed THI was found to be 84.5 (1 h on farm 3) considering all days. Therefore, cows in this study were assumed to be exposed to just moderate heat stress defined as a THI ranging from to 67 to 86 (Zimbelmann et al., 2009; Ortiz et al., 2015). However, the relationship between daily maximum THI instead of daily mean THI per day and concentrations of 11,17-DOA was calculated. Similar results to the results of calculations using daily mean THI were found. The THI used in this study was similar to one out of seven THIs that were compared in another study (Bohmanova et al., 2007). They found differences in thresholds of heat stress among indices and between regions. The THI used in this study is consistent with the THI in their study representing the best index for detection of heat stress in semiarid climate with a lower relative humidity. All sites of this study were located in the temperate climate zone. Therefore, we assume the THI used in our study to be an appropriate heat load index regarding the study of Bohmanova et al. (2007).

However, there were some limitations regarding our study. First, THI in the barn instead of physiological parameters such as respiration rate to assess heat stress was used for logistical reasons. It is well documented that heat stress in dairy cows increases rectal temperature and respiration rate (Bernabucci et al., 2002; Kadzere et al., 2002; De Rensis and Scaramuzzi, 2003; West, 2003; Schütz et al., 2010). This was also the case when mild to moderate heat stress occurred (Ortiz et al., 2015) or when dry cows with a lowered metabolic rate were compared to lactating cows (do Amaral et al., 2009; Thompson et al., 2014). However, a direct relationship between heat stress and individual parameters has not been shown. Therefore, it was assumed for this study that 1) an association between THI as a parameter for heat stress and vital parameters existed, 2) an individual assessment would not yield added value, and 3) THI measured in the pen via a climate logger attached near the ground (3 m) is representative of the heat stress an individual cow experiences as previously shown by Schüller et al. (2013). Future research is warranted to assess the validity and

practicality of respiration rate and rectal temperature as indicators of an individual cow's heat stress. Second, measuring 11,17-DOA might not be the quickest or cheapest method to verify stress. However, collecting fecal samples for 11,17-DOA analysis can be easily conducted without stressing the animal (Palme, 2012) and determining 11,17-DOA in cows under heat stress seems important to us. This study is assumed to help optimizing the validity of 11,17-DOA in following studies when the effect of heat stress on 11,17-DOA concentrations can be considered. Third, we are aware of potential biasing factors on 11,17-DOA values due to the study design i.e., five farms undergoing various periods of heat stress. Using both statistical approaches, the factor farm did have an influence on fecal 11,17-DOA concentrations in cows without heat stress ($P < 0.001$). Nevertheless, we set out to study the relationship between THI and 11,17-DOA under field conditions. Also, there was little variation in mean daily THI or in the number of days with heat stress among farms 1 to 4. Therefore, we assumed the magnitude and duration of heat stress on these four farms with heat stress to be comparable. More controlled studies could be useful to underline the fact that fecal 11,17-DOA is responsive to and a reliable and quantifiable indicator of heat stress in dairy cows. These studies should include comparison of multiple cows not experiencing heat stress and then consistently experiencing elevated THI at the same time.

The hypothesis could be confirmed that acute heat stress is associated with higher fecal 11,17-DOA concentration. This was, however, not the case in cows under chronic heat stress. Independent of the statistical approach, fecal 11,17-DOA concentrations of late-lactation cows without and with chronic heat stress did not differ. Therefore, concentrations of fecal 11,17-DOA can be assumed to be a reliable indicator of acute heat stress in dairy cattle. Additionally, the factor farm affected 11,17-DOA concentrations ($P < 0.001$). Individual variations in cortisol metabolism could have caused these differences (Palme et al., 2000; Morrow et al., 2002). Additionally, farm management differences or other environmental factors may account for a within farm variation. However, further studies are recommended to test if 11,17-DOA could be used as a parameter to quantify acute heat stress in early- and mid-lactation cows, which are often challenged by metabolic disorders.

We are aware of the fact that no specific reproduction data are presented here. Nevertheless, heat stress represents one important influential factor on the reproductive performance of dairy cows (e.g. West, 2003). Validity of future studies, which use fecal 11,17-DOA concentrations, could be increased by including heat stress as a biasing variable in statistical analysis.

4.6 Conclusions

Higher concentrations of 11,17-DOA were associated with acute heat stress. Thus, 11,17-DOA may be used as a parameter for acute heat stress in individual dairy cows particularly as a tool in research on stress responses rather than a direct parameter assessed on-farm.

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4.8 Conflict of interest statement

None of the authors have any conflict of interest to declare.

4.9 Author contributions

A Rees, C Fischer-Tenhagen, W Heuwieser contributed to the design of the study. A Rees collected all samples and analyzed the data. A Rees, C Fischer-Tenhagen, W Heuwieser, contributed to the manuscript and approved the final version to be published.

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5 DISCUSSION

The objective of this thesis was to evaluate non-invasive cow-side parameters for the objective diagnosis of clinical mastitis via udder firmness and for the verification of heat stress via fecal cortisol metabolites in dairy cows. Parameter evaluation set out to improve objective measurement of animal welfare as stipulated by research and the general public (e.g., von Keyserlingk et al., 2009). Specifically, the objectives were 1) to evaluate the validity of estimates of udder firmness determined by manual palpation and a dynamometer and to compare a 4-point palpation scoring system with data obtained with the dynamometer (considering within-observer repeatability, between-observer repeatability, time of measurement, day of study), 2) to investigate if udder firmness can be used as a cow-side indicator for mastitis, and 3) to evaluate if acute and chronic heat stress in individual dairy cows is associated with concentrations of fecal 11,17-DOA.

The results of the first study demonstrated that udder firmness could be measured repeatably by palpation and with the dynamometer, especially when performed by a single observer (within-observer repeatability). When different observers assessed udder firmness, averaged measurements by palpation and with the dynamometer showed high repeatability (between-observer repeatability), respectively. Single measurements obtained by palpation were moderately correlated between observers. Houe et al. (2002) demonstrated poor agreement of udder examination when classifying non-pathological conditions such as hardness of the udder parenchyma determined by palpation. In contrast to their study, measurement procedure in this study was highly standardized (i.e., fingertips were applied at a defined place of udder), whereas in their study the whole udder was palpated, which bears a greater variability. Single measurements performed with the dynamometer were moderately correlated between observers. These data are contrary to a previous study (Bertulat et al., 2012) in which a high between-observers repeatability was described. A smaller number of observers (two vs. nine), or biasing conditions such as movement of cows between measurements and variation in ankle joint position (Österman and Redbo, 2001) can help explain this discrepancy. These results indicated further that a single classification of udder firmness on a 4-point scale obtained by palpation, carried out once and conducted by different observers, was more comparable than measurements obtained by dynamometer by different observers. Between-observer repeatability of average measurements of both measuring methods, however, was similar. Therefore, these results provided evidence that only a highly standardized protocol for udder examination will lead to reliable and repeatable results.

Time of measurements (before and after milking) had a substantial influence on the values of udder firmness. Therefore, udder firmness measurements should be conducted at a consistent time of the day and after milking as indicated by a good repeatability of

measurements in general and a higher correlation between palpation and dynamometer after milking compared to correlation before milking. This finding substantiates the recommendation of a previous study conducted by our group (Bertulat et al., 2012) and provides science-based information for descriptions in textbooks (Rosenberger et al., 1990; Radostits et al., 2001), which advocate an examination after milking. It can be speculated that the volume of the milk or the weight effect on the udder suspension system could have affected measurements.

Despite the subjective nature of palpation, it meets the criterion of a practical diagnostic approach, as it is fast and feasible to assess (Vasseur et al., 2015). Association of estimates of udder firmness on the 4-point scoring system obtained by palpation and with the dynamometer, however, was moderate. Movements of the cow between two measurements leading to different postures of the hind legs relative to the udder and influencing udder firmness could be one explanation for the poor relationship.

Correlation of the two measuring methods did not improve over time. However, a constant Spearman rank correlation coefficient over the days of the trial indicated that in-vitro training of observers could have reduced variation, and thus have contributed to a more consistent diagnostic performance. This observation differed from the results of an earlier study (Houe et al., 2002) that found increased κ correlation values for examinations conducted on different days. Those authors assumed an effect of practicing clinical examination.

It is noteworthy that while a reduction of within- and between-observer variation of animal-based measures is highly important (Rushen et al., 2011; EFSA, 2012; Gibbons et al., 2012; Vasseur et al., 2015), studies comparing multiple diagnostic methods to examine udder health are scarce. To fill this gap and to evaluate udder firmness in cows with CM, a follow-up study (additional unpublished work, second study) was conducted to investigate if udder firmness can be used as a cow-side indicator for CM. To my knowledge, beside one preliminary trial (Rees et al., 2013), this has been the first study on udder firmness in cows with CM using a validated device. Thus, beside a device to assess changes in pain sensitivity in the course of a CM i.e., a pressure algometer (Fitzpatrick et al., 2013), the dynamometer would be the first measuring device evaluating a symptom of the gland itself to diagnose CM in addition to milk-based detection systems such as cell count or conductivity.

Results of the second study clearly indicated that firmness in quarters with CM was higher compared to quarters without CM. An increase of udder firmness of the quarter with CM did not affect the firmness of its healthy neighboring quarter, as firmness of healthy quarters did not differ within or between all cows. Thus, a healthy quarter next to a quarter with CM can be used as a negative control on the quarter level. However, to reduce grouping of data (data clustering) and to provide a parameter on the cow level by using the cow as her own negative

control, Δ firmness (difference in udder firmness between both hind quarters) was calculated and used for further analyzes. After milking, cows with CM had a higher Δ firmness compared to healthy cows due to one firmer udder quarter with CM. Considering receiver operating characteristic curves, Δ firmness provided a very accurate test (Swets, 1988; Ospina et al., 2010) for the differentiation between cows without and with CM. A Δ firmness threshold of > 0.282 kg and > 0.425 kg provided the best combination of sensitivity and specificity for cows in their first parity and for older cows, respectively. Specifically, 64.3% of first-parity cows and 62.5% of older cows were identified as truly suffering from CM using these thresholds. Sensitivity of the method is of particular importance in mastitis detection (Pyörälä, 2003), as improved sensitivity of a mastitis detection technique improves herd udder health (Kamphuis et al., 2010). On the other hand, the evaluation method in milking systems should be highly specific for healthy quarters to avoid separating normal milk (Hovinen and Pyörälä, 2011) and potentially treating healthy cows with antibiotics. However, similar to other methods (Rutten et al., 2013), Δ firmness thresholds provided either a high sensitivity or high specificity, but could not achieve both of the target values (sensitivity: 80%, specificity: 99%) set by the ISO (International Organization for Standardization, 2007).

Infected quarters were firmer until the end of the observation period of 14 days, indicating a healing process of greater than two weeks. These results are consistent with recently published data suggesting that the recovery period after a CM expressed as reduced milk yield, elevated lactate dehydrogenase activity, lower milking frequency and elevated inter-quarter yield ratio continued for weeks after the antibiotic treatment (Fogsgaard et al., 2015).

Parity significantly affected Δ firmness on D0 and on all following days. Regardless of CM, older cows had a higher Δ firmness compared to first-parity cows on all study days. While Δ firmness of older cows was further influenced by milk yield per day and DIM, Δ firmness in first-parity cows was only influenced by CM. This could be explained by 1) the smaller udders of young cows (Fossing et al., 2006), 2) the sustained growth of parenchymal and alveolar cells in the udder until fifth or sixth parity (Klaas et al., 2004), 3) an assumed missing bias through previous cases of CM in first-parity cows, and 4) the different shape of lactation curves in older cows (Horan et al., 2005) resulting in higher variation of milk yield within one lactation, which affects udder firmness.

Severity of CM, defined as the absence or presence of systemic signs of sickness, influenced udder firmness throughout the 14 days. Cows with mild to moderate CM showed lower Δ firmness compared to cows with severe CM. This indicates that cows with systemically signs of a CM also suffer from a firmer udder. Furthermore, bacteriological cure rate was lower in cows with severe mastitis (25%) compared to cows with mild to moderate CM (52%). This finding is similar to previous observations showing that the probability of bacteriological cure

decreases when the cow is systemically ill (Steeneveld et al., 2011). As only a limited number of CM cases (n = 45) from one herd was included, however, the study population may not represent all variations and clinical signs of CM (Hovinen and Pyörälä, 2011).

We could not confirm the findings of Fasulkov et al. (2012) and Oliveira et al. (2013), that gram-negative pathogens cause more pronounced changes in echogenicity of udder tissue and more severe symptoms than gram-positive pathogens regarding udder firmness, respectively. This might be due to the relative small number of CM caused by gram-negative pathogens (n = 10).

In conclusion, assessing udder firmness is a useful tool to distinguish between healthy and mastitic cows. To optimally diagnose CM, udders of cows should be examined after milking in the middle or lower part of a given udder quarter. Different thresholds for first-parity and older cows should be used to reduce bias by parity. Further research is warranted to evaluate potential confounders such as incomplete emptying of the udder after milking (Hovinen and Pyörälä, 2011). Also, it needs to be studied if the firmness of front quarters could serve as a diagnostic tool likewise. It remains unclear if udder firmness can be used as a predictor for the prognosis of a CM or the cure of inflammation.

The second parameter investigated in this thesis was glucocorticoid metabolites as an indicator for heat stress of dairy cows measurable in feces. Specifically, the objective of the third study was to investigate if concentrations of fecal 11,17-DOA could be used to assess a possible stress response i.e., adrenocortical activity of dairy cows exposed to heat stress. To my knowledge, this was the first study evaluating this approach.

Stress assessment measurements need to be non-invasive and sufficiently benign so as not to confound the assessment of stress levels (Morrow et al., 2002). While this may not be the case when e.g., blood is sampled to measure levels of blood cortisol, sampling feces can be conducted without stressful restraining and manipulating the cow. Thus, measuring fecal cortisol metabolites offers a feedback-free method (Möstl and Palme, 2002), which met my aim of using animal-friendly sampling methods.

The hypothesis could be confirmed that acute heat stress is associated with higher concentrations of fecal 11,17-DOA in late-lactation cows. This was, however, not the case in cows under chronic heat stress as concentrations of fecal 11,17-DOA in cows without and with chronic heat stress did not differ. Therefore, concentrations of fecal 11,17-DOA can be assumed to be a reliable indicator of acute heat stress in dairy cattle. These findings confirm results of previous studies on glucocorticoids (Christison and Johnson, 1972; Alvarez and Johnson, 1973; Silanikove, 2000). The decline in hormone concentration under heat stress could be explained by a general attempt to reduce metabolic heat production via reduced

concentration of key metabolic hormones (West, 2003; Bernabucci et al., 2010). Additionally, the factor farm significantly affected 11,17-DOA concentrations. Individual variations in cortisol metabolism (Palme et al., 2000; Morrow et al., 2002) and variations in farm management or other environmental factors could have caused these differences and may have accounted for a within-farm variation. However, to minimize bias of concentrations of 11,17-DOA due to transportation (Palme et al., 2000), handling (Pesenhofer et al., 2006), high metabolic activity (Wiltbank et al., 2006) and milk yield (Bertulat et al., 2013), only late-lactation and healthy cows with similar milk production and stage of lactation were included. Further studies are recommended to test if 11,17-DOA could be used as a parameter to quantify acute heat stress in early- and mid-lactation cows which are often challenged by metabolic disorders possibly biasing concentrations of 11,17-DOA.

This study confirms previous observations (Silanikove, 2000; Hammami et al., 2013; Van laer et al., 2014) that heat stress also affects cows housed in temperate latitudes. Variation in climate as occurring in this study might challenge cows more intensively compared to continuously hot conditions due to a lacking adaptation to permanently hot climates (Kadzere et al., 2002).

Validity of future studies, which use fecal 11,17-DOA concentrations to evaluate other stressful situations, could be increased by including heat stress as a biasing variable in statistical analysis. Future research is encouraged to assess the validity and practicality of respiration rate and rectal temperature as indicators of an individual cows' heat stress considering concentrations of 11,17-DOA.

Overall, validity of both parameters was verified. Udder firmness can be measured repeatably and provides a feasible and easy to use indicator for CM. Concentrations of fecal glucocorticoid metabolites were proven to be affected by acute heat stress. Overall, results of the three studies contribute to the improvement of objective cow-side tests to detect important conditions negatively affecting animal welfare of dairy cows.

6 SUMMARY

Evaluation of udder firmness and fecal cortisol metabolites as cow-side parameters in dairy cows

Animal welfare issues of food-producing animals are of increasing significance both in research and to the general public. As a result, there is a pressing need for the evaluation of non-invasive, practical, cow-based and standardized parameters to objectively measure animal welfare and stress in dairy cows on-farm. Data regarding validity and reliability of these measures is crucial. A high level of standardization in parameter measurement also leads to a more specific and optimized treatment of a disease.

The focus of this work was to evaluate non-invasive cow-side parameters to objectively verify clinical mastitis (CM) via udder firmness and heat stress via fecal glucocorticoid metabolites (11,17-dioxoandrostanes; 11,17-DOA). Both CM and heat stress negatively affect the welfare of dairy cows, result in behavioral changes such as lowered lying time and impair the reproductive performance. Furthermore, both cause substantial economic losses. Therefore, these topics are highly important for the dairy industry and intensively investigated.

Assessment of udder firmness is an essential part of a sound clinical examination of a dairy cow and a practical tool to detect CM promptly. Therefore, validity and reliability of estimates of udder firmness generated by palpation and by using a validated dynamometer in healthy lactating dairy cows was evaluated in the first study. Specifically, this study set out to determine within-observer repeatability and between-observer repeatability expressed as the intraclass correlation (ICC) in two specific experiments. Additionally, a 4-point palpation scoring system was compared with estimates obtained with a dynamometer.

First, the range of udder firmness of 25 cows was determined and an in-vitro model for udder firmness was developed in a pilot trial. This model enabled training of the observers and allowed investigating a 4-point palpation scoring system. In vivo, udder firmness was determined before and after milking by palpation and by using a dynamometer.

In experiment 1, within-observer repeatability based on estimates of udder firmness of 25 cows obtained by three observers on a single day (n = 500) by palpation was 0.968 [95% confidence interval (CI): 0.960 to 0.975]. Within-observer repeatability of these estimates of udder firmness obtained with the dynamometer was 0.997 (95% CI: 0.996 to 0.998) with a coefficient of variation of 9.1% (mean $\pm$ SD: 1.176 $\pm$ 0.107 kg). To determine between-observer repeatability, udder firmness of 100 cows was measured on four different days by nine observers in experiment 2. Considering all measurements (n = 1,800), between-observer repeatability of estimates of udder firmness obtained by palpation and the dynamometer was 0.932 (95% CI: 0.917 to 0.945) and 0.898 (95% CI: 0.867 to 0.925), respectively. Thus, udder

firmness in dairy cows could be measured repeatably with both methods, especially when performed by a single observer. Estimates of udder firmness generated by palpation and with the dynamometer were moderately related (correlation coefficient: 0.54; n = 3,600; $P < 0.001$). Training of observers through the pilot trial or practical experience in the four days of the study in experiment 2 did not improve the correlation. As only healthy cows were included in this first study, further research was warranted to understand how udder firmness develops in infected udders. Therefore, a follow-up study (additional unpublished work, second study) focused on udder firmness in cows suffering from CM.

Swelling and an increased firmness of the mammary gland is an important sign to detect mastitis in dairy cows. The overall objective of this study was to evaluate if udder firmness can be used as a cow-side indicator for CM. The dynamometer was used to objectively determine udder firmness before and after milking in 45 cows with CM and 95 healthy cows. Udder firmness of both hind quarters was measured daily on three locations (upper, middle, lower measuring point) from the day of mastitis diagnosis till day 7 and again on day 14. Firmness of the middle measuring point was highest before and after milking in all cows ($P < 0.001$). Udder firmness before milking was similar ($P = 0.675$) in quarters without [1.867 kg, interquartile range (IQR): 1.243 to 2.671 kg; n = 6,340] and with CM (1.948 kg, IQR: 1.213 to 2.819 kg; n = 1,220). Subsequently, we concentrated on firmness measured on the middle point after milking. After milking, quarters with CM were firmer than healthy quarters ($P < 0.001$). An increase of firmness in a quarter with CM did not affect firmness in the healthy neighbor quarter ($P = 0.419$) nor did firmness of all healthy quarters differ ($P = 0.349$ to 0.931). To reduce grouping of data (data clustering), one firmness value per cow i.e., Δ firmness (difference in udder firmness between both hind quarters), was used for all further calculations. In all cows, CM affected Δ firmness significantly. More precisely, Δ firmness differed between cows without (0.098 kg, IQR: 0.030 to 0.216 kg) and with CM (0.756 kg, IQR: 0.170 to 1.914 kg; $P < 0.001$). In cows in their second and greater parity, Δ firmness was also significantly affected by milk yield per day and DIM.

The threshold for detection of CM using Δ firmness was 0.282 kg (area under the curve: 0.722; sensitivity: 64.3%, specificity: 89.7%) and 0.425 kg (area under the curve: 0.817; sensitivity: 62.5%, specificity: 96.7%) in cows in their first parity and older cows, respectively.

Cows with CM had a higher Δ firmness compared to cows without CM throughout the 14 days after the mastitis diagnoses ($P < 0.001$). Parity had an effect on Δ firmness ($P = 0.016$). Depending on the absence or presence of systemic signs of sickness, cows with CM were classified into cows with mild to moderate (n = 21) or severe CM (n = 24). Bacteriological and clinical cure was defined based on two bacteriological negative milk samples taken at day 7 and 14 after enrollment and normal appearance of milk on the same days. Cows with severe

CM suffered from a firmer udder on all measuring days ($P < 0.001$). An effect of parity ($P = 0.140$) and bacteriological cure ($P = 0.262$) on Δ firmness did not exist. Cows not clinically cured showed an increased Δ firmness of 0.560 kg compared to cured cows ($P < 0.001$).

In conclusion, udder firmness can be a useful indicator for CM in hind quarters. Further research is warranted to evaluate if firmness estimated by palpation and firmness in front udders could also be used as an indicator for CM. It also remains unclear if udder firmness can be used as a predictor for the prognosis of a CM.

Measurement of fecal cortisol metabolites in fecal samples is a scientifically established and practical method to determine stress levels in animals. In dairy cows, fecal cortisol metabolites have already been used as an indicator for stress during the transition period, dry-off, handling and transport. In this thesis, the focus is on a new field of application of measurement of fecal cortisol metabolites i.e., measurement of heat stress. The negative impact of heat stress on health and productivity of dairy cows is well known. Heat stress can be quantified with the temperature-humidity-index (THI) and is defined as a THI $\geq$ 72. For aforementioned reasons, finding a way to quantify heat stress in dairy cows has been of increasing interest over the past decades. Therefore, the objective of the third study of this thesis was to evaluate concentrations of fecal glucocorticoid metabolites (i.e., 11,17-dioxoandrostanes; 11,17-DOA) as an indirect stress parameter in dairy cows without heat stress (DOA 0), with heat stress on a single day (acute heat stress; DOA 1) or with more than a single day of heat stress (chronic heat stress; DOA 2).

Cows were housed on five farms under moderate European climates. Two statistical approaches (approach 1 and 2) were assessed. Using approach 1, concentrations of fecal 11,17-DOA were compared among DOA 0, DOA 1 and DOA 2 samples regardless of their origin (i.e., cow) using an unpaired test. Using approach 2, a cow was considered as its own control i.e., 11,17-DOA were treated as a cow-specific factor and only paired samples were included in the analysis for this approach (paired tests). In approach 1 ($P = 0.006$) and approach 2 ($P = 0.038$) 11,17-DOA values of cows under acute heat stress were higher compared to those of cows without heat stress. Therefore, the hypothesis could be confirmed that acute heat stress is associated with higher fecal 11,17-DOA concentration. This was, however, not the case in cows under chronic heat stress as concentrations of fecal 11,17-DOA in cows without and with chronic heat stress did not differ. Results of this study indicate that acute heat stress has to be considered as a confounder in studies measuring fecal glucocorticoid metabolites in cows to evaluate other stressful situations.

Regarding the overall hypothesis of my thesis, validity of both parameters was verified. Udder firmness can be measured repeatably and provides a feasible and easy-to-use indicator for CM. To optimally diagnose CM, udder of cows should be examined after milking in the middle or lower part of a given udder quarter. Furthermore, different udder firmness thresholds should be used to diagnose CM in first-parity or older cows. Concentrations of fecal glucocorticoid metabolites were proven to be affected by acute heat stress. Overall, results of the three studies add one part to the improvement of objective cow-side tests to detect important conditions negatively affecting animal welfare of dairy cows.

7 ZUSAMMENFASSUNG

Evaluierung von Euterfestigkeit und der Konzentration von Kortisolmetaboliten im Kot als mögliche kuhbasierte Parameter bei Milchkühen

Das Wohlergehen lebensmittelliefernder Tiere ist sowohl in der Forschung als auch für die Öffentlichkeit von zunehmender Bedeutung. Daher besteht eine zwingende Notwendigkeit zur Evaluierung geeigneter, kuhbasierter und standardisierter Parameter zur objektiven Messung von Tierwohl und Stress bei der Milchkuh direkt auf dem Betrieb. Daten zu Validität und Zuverlässigkeit dieser Messungen sind essentiell. Ein hohes Maß an Standardisierung führt auch zu spezifischeren und optimierten Behandlungen von Erkrankungen.

Der Fokus dieser Arbeit lag auf der Evaluierung nicht-invasiver und kuhbasierter Parameter, um klinische Mastitis (CM) mittels Euterfestigkeit und Hitzestress mithilfe von im Kot nachweisbaren Kortisolmetaboliten (11,17-Dioxoandrostane; 11,17-DOA) objektiv zu messen. Sowohl CM als auch Hitzestress haben einen negativen Effekt auf das Tierwohl der Milchkühe, führen zu Verhaltensveränderungen wie beispielsweise Verringerung der Liegezeit und beeinträchtigen die Reproduktionsleistung. Zusätzlich führen beide zu erheblichen wirtschaftlichen Verlusten. Daher sind sie von hoher Wichtigkeit für die Milchindustrie und werden intensiv erforscht.

Beurteilung der Euterfestigkeit ist essentieller Bestandteil einer sorgfältigen klinischen Untersuchung der Milchkuh und eine geeignete Methode zur direkten Erkennung der CM. Aus diesem Grund wurden Validität und Zuverlässigkeit von Messungen der Euterfestigkeit gesunder laktierender Milchkühe mittels Palpation und einem validiertem Dynamometer in einer ersten Studie evaluiert. Dazu wurde die Wiederholbarkeit von Messungen innerhalb eines Untersuchers und zwischen verschiedenen Untersuchern in zwei Experimenten bestimmt und durch den Intraklassen-Korrelationskoeffizient (ICC) ausgedrückt. Zusätzlich wurde eine 4-Punkte-Palpationsskala mit den durch das Dynamometer ermittelten Werten verglichen.

Zunächst wurde im Rahmen einer Pilotstudie der Wertebereich der Euterfestigkeit bei 25 Kühen bestimmt und ein In-vitro-Euterfestigkeitsmodell entwickelt. Dieses Modell ermöglichte ein Training der Untersucher und erlaubte die Entwicklung einer 4-Punkte-Palpationsskala. Die Euterfestigkeit wurde in vivo durch Palpation und Dynamometer vor und nach dem Melken gemessen. In Experiment 1 betrug die Wiederholbarkeit von Euterfestigkeitsmessungen innerhalb eines Untersuchers, die mittels Palpation bei 25 Kühen und durch drei Untersucher an einem einzelnen Tages durchgeführt wurden (n = 500), 0,968 [95% Konfidenzintervall (CI): 0,960 bis 0,975]. Die Wiederholbarkeit dieser Messungen der Euterfestigkeit mit dem Dynamometer innerhalb eines Untersuchers war 0,997 (95% CI: 0,996

bis 0,998) und wies einen Variationskoeffizient von 9,1% (Mittelwert ± Standardabweichung: 1,176 ± 0,107 kg) auf. Um die Wiederholbarkeit von Messungen zwischen verschiedenen Untersuchern zu ermitteln, wurde in Experiment 2 die Euterfestigkeit bei 100 Kühen an vier verschiedenen Tagen durch neun Untersucher gemessen. Unter Berücksichtigung aller Messungen (n = 1.800), betrug die Wiederholbarkeit der Messungen mittels Palpation zwischen den Untersuchern 0,932 (95% CI: 0,917 bis 0,945) und 0,898 (95% CI: 0,867 bis 0,925) bei Messungen mit dem Dynamometer. Folglich konnte die Euterfestigkeit bei Milchkühen, vor allem wenn sie durch einen einzelnen Untersucher ermittelt wurde, durch beide Methoden wiederholbar gemessen werden. Euterfestigkeitswerte, welche durch Palpation und Dynamometer ermittelt wurden, waren mit einem Korrelationskoeffizienten von 0,54 (n = 3.600; $P < 0,001$) moderat korreliert. Diese Korrelation konnte durch einen Trainingseffekt durch die Pilotstudie oder praktische Erfahrung in den vier Tagen der Studie nicht verbessert werden. Da in dieser Studie ausschließlich gesunde Kühe untersucht wurden, war eine weitere Studie zur Entwicklung der Euterfestigkeit in entzündeten Eutervierteln notwendig. Daher lag der Schwerpunkt einer Folgestudie (zusätzliche unveröffentlichte Daten, zweite Studie) auf der Untersuchung der Euterfestigkeit bei Kühen, die an CM erkrankt waren.

Die Schwellung und Verfestigung der Milchdrüse gehört zu den klassischen Entzündungszeichen einer Mastitis und ist daher ein wichtiger Befund zur Erkennung der CM in Milchkühen. Das Hauptziel dieser Studie war es, Euterfestigkeit als einen kuhbasierten Indikator für CM zu validieren. Dazu wurde bei 45 Kühen mit CM und bei 95 gesunden Kühen die Euterfestigkeit vor und nach dem Melken mit dem Dynamometer gemessen. Die Festigkeit beider Hintervierteil wurde täglich an je drei Punkten (oberer, mittlerer und unterer Messpunkt) beginnend am Tag der Mastitisdiagnose (Tag 0) bis zum Tag 7 und an Tag 14 gemessen. Die Festigkeit des mittleren Messpunktes war bei allen Kühen sowohl vor als auch nach dem Melken am höchsten ($P < 0,001$). Vor dem Melken glich sich die Euterfestigkeit gesunder [1,867 kg, Interquartilsabstand (IQR): 1,243 bis 2,671 kg; n = 6.340] und erkrankter Viertel (1,948 kg, IQR: 1,213 bis 2,819 kg; n = 1.220; $P = 0,675$). Für die weitere Auswertung wurde ausschließlich die nach dem Melken gemessene Festigkeit des mittleren Messpunktes berücksichtigt. Nach dem Melken waren an CM erkrankte Viertel fester als gesunde ($P < 0,001$). Die Festigkeit aller gesunden Viertel unterschied sich nicht ($P = 0,349$ bis 0,931) und wurde nicht durch die Verfestigung des entzündeten Nachbarviertels beeinflusst ($P = 0,419$). Um die Gruppierung von Daten (Datencluster) zu reduzieren, wurde für alle folgenden Berechnungen ausschließlich ein einziger Festigkeitswert pro Kuh, Δ Festigkeit (Differenz der Euterfestigkeit zwischen beiden Hintervierteln in kg), verwendet. Bei allen Kühen beeinflusste eine CM die Δ Festigkeit signifikant. Genauer gesagt unterschied sich Δ Festigkeit von gesunden (0,098 kg, IQR: 0,030 bis 0,216 kg) und erkrankten Kühen (0,756 kg, IQR: 0,170 bis

1,914 kg; $P < 0,001$). Bei Kühen in der zweiten oder höheren Laktation wurde Δ Festigkeit weiterhin durch die Tagesmilchleistung und die Tage in Milch signifikant beeinflusst.

Der Grenzwert für die Erkennung einer CM mittels Δ Festigkeit betrug 0,282 kg (Fläche unter der Kurve: 0,722; Sensitivität: 64,3%, Spezifität: 89,7%) für Erstlaktierende und 0,425 kg (Fläche unter der Kurve: 0,817; Sensitivität: 62,5%, Spezifität: 96,7%) für Kühe in der zweiten oder höheren Laktation.

Kühe mit CM hatten im Verlauf der 14 Tage nach der Mastitisdiagnose stets eine höhere Δ Festigkeit als gesunde Kühe ($P < 0,001$). Die Laktationszahl beeinflusste auch hier Δ Festigkeit ($P = 0,016$). In Abhängigkeit von dem Vorhandensein systemischer Krankheitsanzeichen wurden die an CM erkrankten Kühe aufgeteilt in Kühe mit milder und moderater (n = 21) oder Kühe mit schwerer CM (n = 24). Kühe mit CM, bei denen zwei bakteriologisch negative Milchproben an Tag 7 und 14 gewonnen wurden, galten als bakteriologisch geheilt. Als klinisch geheilt galten Kühe, die an diesen Tagen keinerlei Abweichungen vom Milchcharakter aufwiesen. Kühe mit schwerer CM litten an allen Messtagen an einem festeren Euter ($P < 0,001$). Ein Effekt der Laktationszahl ($P = 0,14$) oder der bakteriologischen Heilung ($P = 0,262$) auf Δ Festigkeit konnte nicht festgestellt werden. Kühe ohne klinische Heilung hatten eine um 0,560 kg höhere Δ Festigkeit als klinisch geheilte Kühe ($P < 0,001$).

Zusammenfassend kann gesagt werden, dass die Euterfestigkeit ein geeigneter Indikator zur Erkennung der CM in den Hintervierteln darstellt. Weiterführende Untersuchungen sind nötig um zu eruieren, inwiefern die Festigkeit der Vorderviertel sowie die durch Palpation gemessene Festigkeit zur Erkennung einer CM genutzt werden kann. Weiterhin unklar ist auch, inwiefern Euterfestigkeit als prädiktive Größe für die Prognose einer CM genutzt werden kann.

Die Messung von Kortisolmetaboliten in Kotproben ist eine wissenschaftlich etablierte und praktikable Methode für die Bestimmung von Stresszuständen in Tieren. Im Kot nachweisbare Kortisolmetabolite wurden bereits dazu genutzt, Stress bei Milchkühen während der Transitperiode, während des Trockenstellens, beim Handling und beim Transport nachzuweisen. Der Fokus dieser Arbeit lag auf der Messung von Hitzestress mittels der Konzentration von Kortisolmetaboliten im Kot, einem neuen Anwendungsgebiet dieser Methode. Der negative Einfluss von Hitzestress auf die Gesundheit und Leistung von Milchkühen ist gut beschrieben. Hitzestress wird durch den Temperatur-Feuchtigkeits-Index (THI) quantifiziert und ist dann definiert als ein THI $\geq$ 72. Aus oben genannten Gründen besteht seit einigen Jahrzehnten wachsendes Interesse an der Quantifizierung von Hitzestress. Daher bestand das Hauptziel der dritten Studie dieser Arbeit darin, die Konzentration von

Glukokortikoidmetaboliten im Kot (11,17-Dioxoandrostane; 11,17-DOA) als einen indirekten Stressparameter bei Milchkühen ohne Hitzestress (DOA 0), mit Hitzestress an einem einzigen Tag (akuter Hitzestress, DOA 1) oder mit über einem Tag Hitzestress (chronischer Hitzestress, DOA 2) zu evaluieren.

Die Kühe wurden auf fünf Betrieben und unter gemäßigtem europäischem Klima gehalten. Zwei statistische Ansätze (Ansatz 1 und 2) wurden angewandt. In Ansatz 1 wurden die 11,17-DOA-Konzentrationen im Kot zwischen DOA 0-, DOA 1- und DOA 2-Proben ungeachtet ihrer Herkunft (Kuh) mittels ungepaartem Test verglichen. Für Ansatz 2 wurde jede Kuh als ihre eigene Kontrolle berücksichtigt. Folglich wurde 11,17-DOA als kuhspezifischer Faktor behandelt und ausschließlich gepaarte Stichproben in die Berechnungen für diesen Ansatz mit einbezogen (gepaarter Test). Die 11,17-DOA-Konzentration bei Kühen unter akutem Hitzestress war im Vergleich zu Kühen ohne Hitzestress sowohl in Ansatz 1 ($P = 0{,}006$) als auch in Ansatz 2 ($P = 0{,}038$) erhöht. Somit konnte die Hypothese bestätigt werden, dass akuter Hitzestress mit einer erhöhten Konzentration von Kortisolmetaboliten im Kot assoziiert ist. Dies war nicht der Fall bei Kühen, die unter chronischem Hitzestress litten, da sich die 11,17-DOA-Konzentration von Kühen ohne und unter chronischem Hitzestress nicht unterschied. Diese Ergebnisse weisen darauf hin, dass in Studien, welche die Messung von Kortisolmetaboliten im Kot nutzen um Stress zu quantifizieren, akuter Hitzestress als Störvariable berücksichtigt werden sollte.

In dieser Arbeit konnte die Zuverlässigkeit sowohl von Euterfestigkeit als auch von Kortisolmetaboliten im Kot als kuhbasierte Parameter validiert werden. Euterfestigkeit kann wiederholbar gemessen werden und stellt einen praktikablen und einfach zu nutzenden Indikator für CM dar. Um CM optimal diagnostizieren zu können, sollte das Euter nach dem Melken und am mittigen oder unteren Teil des jeweiligen Viertels untersucht werden. Zusätzlich sollten zur Diagnose der CM unterschiedliche Euterfestigkeitsgrenzwerte bei Erstlaktierenden und älteren Kühen genutzt werden. Ein Einfluss von akutem Hitzestress auf die Konzentration von Kortisolmetaboliten im Kot konnte nachgewiesen werden. Zusammenfassend tragen alle drei Studien zur Verbesserung objektiver kuhbasierter Tests bei, um wichtige Zustände, die das Wohlbefinden von Milchkühen beeinträchtigen, zu erfassen.

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9 PUBLICATIONS

Research articles

Rees, A.; C. Fischer-Tenhagen, and W. Heuwieser. (2014):
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11 DECLARATION OF INDEPENDENCE

Hiermit erkläre ich, dass ich, Anne Rees, 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 udder firmness by palpation and a dynamometer

[b] Udder firmness as a possible indicator for clinical mastitis

[c] Effect of heat stress on concentrations of faecal cortisol metabolites in dairy cows

Berlin, den 21.06.2016

Anne Rees