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Effects of an intensive experimental protocol on health, fertility, and production in transition dairy cows

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ABSTRACT

Animal experimentation is required to investigate complex physiological relationships and facilitates development of evidence-based knowledge. However, experimental protocols can interfere with the daily routine of the animals, result in stress and pain, and have adverse effects on health and production. The goal of this study was to investigate the effects of an intensive experimental protocol on health traits and production in transition dairy cows. Eighty experimental dairy Holstein cows (EXP group) underwent serial protocol-based clinical and ultrasonographic examinations, puncture of the jugular vein for blood collection or drug application, and liver biopsy samples, 14 d before until 42 d after parturition. Controls (CTR group) included 206 cows from the same herd, which fulfilled the same inclusion criteria and were kept under the same production management but were not handled for the purpose of this study. Procedure-related effects with a potentially negative effect on health and production were recorded. Furthermore, production, fertility and culling traits of the 2 groups (CTR, EXP) were compared. Most procedure-related adverse effects were associated with transcutaneous liver biopsies and included diffuse inflammation of the skin incision in 11.9% (42 of 320), abscessation of the skin or subcutis in 4.6% (11 of 240), and increased liver echogenicity of the biopsy site in 10.4% (27 of 240). The experimental procedures had a negative effect on milk yield at the start [days in milk (DIM) 5–50, difference: 2.3 kg, standard error (SE): 0.8 kg] and end of lactation (DIM 251–300, difference: 2.0 kg, SE: 1.0 kg; DIM 301–350, difference: 2.3 kg, SE: 1.2 kg) resulting in a lower 305-d milk yield in the EXP group than in the CTR group (difference: 472 kg, SE 214 kg). The milk fat % was higher in the EXP group than in the CTR group from 251 DIM onward (DIM 251–300, difference: 0.20%, SE: 0.09%; DIM 301–350, difference: 0.41%, SE: 0.17%). Also, the somatic cell score was higher in the EXP group than in the CTR group, during early (5–50 DIM, difference: 0.43, SE: 0.22) and from mid-lactation onward (DIM 151–200, difference: 0.43, SE: 0.2; DIM 201–250, difference: 0.49, SE: 0.22; DIM 251–300, difference: 0.55, SE: 0.25; DIM 301–350, difference: 0.61, SE: 0.28). Experimental procedures had no effect on first service conception rate and time to pregnancy, but had a positive effect on stillbirth rate with fewer stillbirths in the CTR group (0%) than in the EXP group (3.9%). Furthermore, experimental handling had no effect on time to culling or type of culling, whereby poor production was a more frequent reason noted for culling in the EXP group. Procedure-associated impairment of production in dairy cows is rarely reported and allows the estimation of the effects of such a study protocol on animal health and production. As a limitation for the interpretation of the results, the number of animals included and conduction in one single herd have to be considered.

Key words: animal experiments, biopsy, stress, manipulation, sampling

INTRODUCTION

Evidence-based knowledge in dairy science is the product of planned, prospective, randomized studies, which generate treatment and management protocols used for decision-making (Vandeweerd et al., 2012). Animal experimentation is strictly regulated by law and is limited to circumstances where it is mandated by regulatory agencies or the use of alternatives is not fea-

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sible. Furthermore, experiments involving animals must be justifiable from an ethical standpoint (European Commission, 2010). In vitro studies are alternatives to live-animal experimentation; however, the simulation of physiologic situations and diseases by means of in vitro systems is not always realistic. Therefore, experiments involving animals still make up a relevant part of studies conducted in life sciences. Of all the animals used in research in Germany in 2019, only 0.27% were cattle (Bundesministerium für Ernährung und Landwirtschaft, 2019). In dairy cattle a main research focus is on the transition period, as it is a time where the animals are especially vulnerable and production diseases predominantly occur. Most cattle are used in applied research studies, many of which were field studies conducted under conditions that reflected production farm settings (Nechanitzky et al., 2016).

Experiments involving live animals vary considerably in their design and extent depending on the objectives of the study but are frequently associated with varying degrees of stress. Bovine studies often include clinical procedures such as transrectal examination (Giese et al., 2018), injections (Abuelo et al., 2016), collection of blood (Kusenda et al., 2013) and urine (Horst et al., 2019), ultrasonographic examination (Starke et al., 2010b), and the collection of rumen fluid (Duffield et al., 2004). It is reasonable to assume that these clinical procedures do not cause as much stress in cattle (Cingi et al., 2012; Smith et al., 2018) as less commonly used but more invasive procedures such as abdominal surgery (Meyer et al., 2007; Mudron et al., 2007; Heppelmann et al., 2015) or the collection of biopsy samples from internal organs (Starke et al., 2010a; Lüttgenau et al., 2011).

The collection of tissue samples, such as liver biopsy for the study of hepatic metabolism, is indispensable for many scientific research objectives. The analysis of liver tissue specimens remains the gold standard for the diagnosis of the fatty liver syndrome (Starke et al., 2010a; Gerspach et al., 2017) and describing liver metabolism (Schären et al. 2021b). Liver biopsy is also used in routine veterinary practice for monitoring trace element status (Charmley et al., 1992; Corah, 1996; Bittner et al., 2021) and for the diagnosis of metabolic disorders (West, 1997; Grünberg et al., 2009; Hammon et al., 2009). It is important to bear in mind that liver biopsy causes stress and potentially pain (Mølgaard et al., 2012) and poses risks such as hemorrhage, inflammation, and inadvertent puncture of an organ other than the liver (Friedman, 2004).

Objective assessment of the severity of a procedure in animals is challenging (Keubler et al., 2020). Severity assessment has been standardized in laboratory animals, where 4 severity classifications have been defined: mild, moderate, severe, and nonrecovery (European Commission, 2012; Smith et al., 2018). Severity categories are usually associated with pain and distress (Balcombe et al., 2004; Castelhano-Carlos and Baumans, 2009). Cattle displayed behaviors consistent with distress and pain after percutaneous needle biopsy of the liver in one study (Mølgaard et al., 2012) but not in another (Barrett et al., 2016). Stress and pain have a negative effect on the health, production, and welfare of cattle (Seabrook, 1972; Hemsworth and Barnett, 1991; Breuer et al., 2000), but the direct effect of experimental procedures on production in dairy cattle has received little attention (Mialon et al., 2012; Khatun et al., 2013). A literature search in PubMed (https://pubmed.ncbi.nlm.nih.gov/) on April 27, 2021, using the key words *animal experiments*, *milk production*, *fertility*, and *cattle* yielded no reports on the impact of complex experimental protocols, involving superimposed and repetitive manipulations of varying severity, on production in healthy transition dairy cattle. Likewise, descriptions of complications associated with serial liver biopsies in individual cows are scant (Swanson et al., 2000). However, knowledge of the effects of intensive experimental manipulations on health and production is paramount when planning studies in farm animals under routine production conditions. During experimental studies often a combination of experimental measures is applied, which have potentially additive negative effects. Knowledge of the overall effect of such an experimental procedure allows a better argumentation during application for animal ethics approval, as until now, for the estimation of study effects, results of studies using laboratory animals had to be included in the argumentation. Furthermore, study results would allow the researcher to provide the farmer with expected health consequences and performance reductions for the experimental animals under such an intensive study protocol.

Therefore, the objective of this study was to investigate the effects of an intensive experimental protocol on health and production traits in transition dairy cows.

MATERIALS AND METHODS

Research Permit

The study was part of an overall project on the "Effect of a metaphylactic treatment of transition dairy cows with vitamin B12 + Butaphosphan (Catosal)." The experiment was conducted in accordance with the

German Animal Welfare Act and the Saxony Veterinary and Food Safety Office Landesdirektion Sachsen, permit number TVV 33/15.

Research Herd

The study was carried out from November 2015 to November 2016 and was conducted in a freestall dairy herd with 660 German Holstein cows (rolling herd average 10,747 kg, 3.73% fat, 3.33% protein as of December 2016) in Saxony, Germany. The mean 305-d milk production of all cows that completed a lactation during the study period was 10,781 kg (range, 4,932 to 15,374 kg; n = 610). The cows were dried off 8 wk before the calculated calving date. Depending on season, dry cows were kept in a straw-bedded barn or on pasture and were moved to a calving pen 1 to 2 wk before the calculated due date. After calving, the cows were housed in a freestall barn with straw-bedded cubicles. After 5 d in a fresh-cow pen, the cows were moved into an early-lactation pen, and further pen changes occurred at 40 and 110 DIM, and later in lactation depending on production level. The cows were fed a TMR and milked twice a day in a double-16 parallel parlor (Lemmer-Fullwood). More detailed description on management, housing, and feeding, see Schären et al. (2021a).

The farm employed a technician, who artificially inseminated all the cows. Pedometers (Pedometer, Lemmer-Fullwood) were used for heat detection. The target voluntary waiting period was 42 d in first-lactation cows and 60 d in older cows but was extended in high-producing cows. In the year preceding the study period (2015), the first service conception rate was 42%, services per conception were 2.6, and the period from first service to conception was 38 d. The stillbirth rate was 14% in first-lactation cows and 5% in multiparous cows for a total stillbirth rate of 8%.

Experimental Cows

The initial list of animals, meeting the inclusion criteria, was based on herd health records entered in the herd management software HerdeW (dsp-Agrosoft GmbH). At enrollment the cows had to have completed a minimum of 1 lactation and had to be 2 wk before the calculated calving date. Cows that had received medical treatment other than dry-cow antibiotics during the 28 d preceding enrollment were excluded. For the experimental study, 87 cows (**EXP** group) were selected from this initial list and subjected to an intensive experimental protocol, which started 14 d before the calculated calving date and ended 49 d after calving. The selection of the animals for the experimental group was based on an even distribution of animals throughout the study period; therefore, not more than 6 cows per day were included in the EXP group. Within the EXP group the treatment assignment was done randomly using a lottery procedure described in Schären et al. (2021a). Seven cows were excluded during the study for reasons that included downer cow syndrome (n = 1), fetal mummification (n = 1), uterine prolapse (n = 1), severe leg injury (n = 1), intractability (n = 1), and misidentification (n = 2). Thus, the number of cows available for analysis in the EXP group was 80.

After completion of the study, surplus animals not included in the study, but meeting the inclusion criteria, were set as control animals (**CTR** group; n = 206). These cows were housed under the same conditions and underwent the same production management as EXP group but they were not handled or monitored for the purpose of this study. The production traits of both groups in preceding lactation (pre-study lactation) are shown in Table 1 and the time to pregnancy for the pre-study lactation in Figure 2A.

Study Design

Table 2 shows the experimental protocol of the cows of the EXP group. On the scheduled days of examination, the cows were brought in pairs and at the same time of day to a straw-bedded holding pen with ad libitum feed and water. This pen was next to the calving pen and out of sight of the production pens. One cow was then moved into an examination chute (Texas Trading Squeeze Chute S04). Depending on the number of procedures scheduled for the day (see Table 2), the cows spent up to 90 min in the chute, during which time they did not have access to feed and water. All experimental procedures were carried out by postgraduate veterinarians who lived at the research farm throughout the study period.

The clinical examination was done as described in Dirksen et al. (2012). Cows were inspected visually in the herd on days that are not specified in Table 2. When clinical abnormalities were seen, the cows underwent a complete physical examination. The cows underwent a final examination on d 49 postpartum, which was 1 wk after the last experimental procedures were carried out (d 42 postpartum).

During the study period, cows received 6 intravenous injections of 5 or 10 mL/100 kg BW of colored isotonic saline solution (group **PLAC**) or 0.005% cyanocobalamin and 10% butaphosphan (**BCC**, Catosal, Bayer Animal Health GmbH) into a jugular vein (group **VER5** and **VER10**). Because the time of calving was difficult to predict, 15 cows did not receive all prepar-

Table 1. Least squares means of the production traits of the pre-study lactation of cows in the experimental (EXP) and control (CTR) group and their differences (EXP − CTR)

Trait	Lactation number	EXP (n = 80) LSM	SE	CTR (n = 206) LSM	SE	Difference (EXP − CTR)	*P*-value
305-d milk yield (kg)	1	9,114	370	8,508	181	606	0.14
	≥2	11,513	207	11,863	143	−350	0.16
Fat (%)	1	3.20	0.10	3.38	0.05	−0.18	0.12
	≥2	3.29	0.05	3.37	0.04	−0.08	0.22
Protein (%)	1	2.84	0.07	2.91	0.03	−0.07	0.32
	≥2	2.83	0.04	2.92	0.02	−0.09	0.04
SCS	1	2.96[1]	Minimum: 1.28 Maximum: 5.42	3.08[1]	Minimum: 0.90 Maximum: 6.31	−0.12	0.75[2]
	≥2	3.25[1]	Minimum: 0.93 Maximum: 6.31	3.43[1]	Minimum: 1.12 Maximum: 6.47	−0.18	0.35[2]
First service conception rate (%)	1	47.4	11.4	50.0	5.60	−2.60	0.84
	≥2	36.1	6.10	40.2	4.30	−4.10	0.59
Stillbirth rate (%)	1	10.5	7.10	12.5	3.70	−2.00	0.80
	≥2	1.60	1.60	6.30	2.20	−4.70	0.08

[1]Arithmetic mean of the original scale.
[2]*P*-value of rank analysis.

tum injections (details in Schären et al., 2021a). Collection of 20 mL of blood was done 8 times per animal and alternated between the left and right jugular veins. A total of 926 manipulations of the jugular vein (blood sampling and injections) were done. Urine was collected during spontaneous micturition or after manual stimulation of the perineum. For B-mode ultrasonographic examination of the liver, the hair on the right side of the cow was clipped from the 7th to the 12th intercostal space and from the level of the stifle to the transverse spinous processes and the skin cleaned with alcohol. After application of conductive gel, the liver was scanned in the standing cow using a convex and a linear 2.5- to 6.6-MHz transducer (MyLabOne Vet, Esaote Europe B.V.). The liver was viewed from the 7th to 12th intercostal spaces. The liver parenchyma was first assessed subjectively by determining the appearance of the surface as well as its echogenicity and pattern. The size of the liver was determined and the echogenicities of the liver and kidney were subjectively compared. The backfat thickness was also measured (Haudum, 2009). Ultrasound-guided liver biopsy was then carried out under local anesthesia with 10 mL of Isocain ad. us. vet. 20 mg/mL (Selectavet) as described in Gohlke et al. (2013); briefly, a stab incision was made in the skin in the 10th or 11th intercostal space using a pointed scalpel, and a custom-made biopsy needle (6 mm outside diameter) was inserted and advanced into the liver. One or two samples were collected (in total 2 g), depending on the size of the first sample. The skin incision was closed with a single suture using nonabsorbable suture material (Supramid 9 EP, SMI, St. Vith). Ultrasonography of the liver and surrounding structures was repeated immediately after the procedure. A total of 320 liver biopsy samples were collected.

Assessment of Adverse Effects Associated with Experimental Procedures

Adverse Effects. In EXP group, observed adverse effects associated with experimental procedures were recorded during clinical and ultrasonographic examinations. Potential complications of the experimental measures and their diagnoses are listed in Table 3. Adverse effects that occurred after a certain time period but not immediately after jugular venipuncture or liver biopsy, such as thrombophlebitis, diffuse inflammation of the skin incision site, abscessation, hematoma, perihepatitis, peritonitis, and increased echogenicity of the biopsy site in the liver, were attributed to the most recent procedure for calculation of the complication rate. Increased echogenicity of the liver biopsy site was defined as an ultrasonographic finding postbiopsy, where the biopsy canal adjacent tissue was more echogenic than

Table 2. Experimental protocol for cows of the experimental group

Time[1]	Examination in chute	Clinical examination	Injection into vena jugularis	Blood sampling vena jugularis	Urine collection	Ultrasonography liver	Liver biopsy
Day peripartum							
−14	+	+		+	+	+	+
−7	+	+	+	+	+	+	
−6	+	+	+				
−5	+	+	+				
Parturition (d)	+	+					
+1	+	+	+	+	+		
+2	+	+	+		+		
+3	+	+	+		+		
+4	+	+			+		
+5	+	+			+		
+6	+	+			+		
+7	+	+		+	+	+	+
+14	+	+		+	+	+	
+21	+	+		+	+	+	
+28	+	+		+	+	+	+
+42	+	+		+	+	+	+

[1]Because of the difficulty predicting the time of parturition or increased workload, the actual sampling times varied a few times from the scheduled times. Minimum and maximum deviations were 1 to 26 d for d −14, 4 to 13 d for d +7, and 23 to 34 d for d +28. These deviations were considered in the analysis.

the rest of the liver tissue. It was included in this list as a possible adverse effect, with so far unknown relevance (see discussion).

According to the study protocol, cows were only examined clinically after the last blood collection and liver biopsy on d 42, and ultrasonography and laboratory examinations were only done when indicated. This means that the occurrence of delayed adverse reactions that required ultrasonography for diagnosis (abscess, hematoma, perihepatitis, and increased echogenicity of the biopsy site in the liver) were analyzed only with respect to the first 3 liver biopsies (n = 240).

Comparison of Production, Fertility, And Culling Traits in Groups EXP and CTR. For this comparison, data that had been entered into the herd management software were analyzed. The following traits were compared:

1. Daily milk production in kg, fat %, and protein %, and SCC, retrieved from the official monthly milk control report by the local state control association (Landeskontrollverband, Sachsen, Germany). According to international standards the SCC was transformed to SCS using the formula SCS = $\log_2$ (SCC/100,000.0) + 3. In general, this results in approximately normally distributed residuals of SCC.
2. Fertility: first service conception rate, time to pregnancy (survival analysis), and stillbirth rate. The trait selection was made from the point of view of an individual animal-related recording, the inclusion of all cows, and thus the possibility of statistical comparison of the groups.
3. Culling rate: overall culling rate, number of cows that were slaughtered, died, or were euthanized, reasons for culling (from the 9 official categories 5 were selected for analysis: mastitis, production, lameness, infertility, and metabolic diseases). Animals that were sold for breeding (CTR n = 2, EXP n = 1) were not included in the calculation of the culling rate. Furthermore, time to culling was included as an analysis trait (survival analysis).

Statistical Analysis

Analysis of Pre-Study Lactation Data. For the group comparison of production, fertility, and culling data the parity was included as fixed lactation class (class 1 with parity 1 and class 2 with parity ≥2) in the analysis of the data of the lactation pre-study period. The comparisons between groups were always made within lactation classes. In the study period we abstained from inclusion of the parity as they were no primiparous cows included in the study, which differ usually in production and fertility data from multiparous cows (Wathes et al., 2007; Mee, 2008).

Analysis of milk production traits in the pre-study lactation was based on a linear model with the fixed effects of groups and lactation class for milk yield, milk fat, and milk protein.

The residuals of SCS in the pre-study lactation did not have a normal distribution and therefore a nonparametric rank analysis was used (Brunner and Munzel, 2013). In case of rank analysis we reported, in addition to the test results, the arithmetic mean in the original

Table 3. Incidence of observed and potential procedure-related adverse effects and their estimated probabilities (interpreted as complication rate %) based on the number of procedures in the experimental group (EXP; n = 80) observed during the experimental period

Adverse effect	Diagnosis	Total number	Complication rate (%)
Possible effect caused by restraint (n = 1,119)			
Injuries sustained in chute	Direct observation	1	0.01
Possible effect of jugular venipuncture (n = 926)			
Jugular thrombophlebitis	Diffuse, warm swelling of the jugular vein region; painful in some cows (Dirksen et al., 2006)	3	0.32
Possible effect of liver biopsy			
Immediate effects (n = 320)			
Inadvertent puncture of another organ	Sample does not resemble liver tissue.	1	0.31
Injury of portal vein	Ultrasonographic observation of the vein puncture	0	0
Hemorrhage	Ultrasonographic visualization of anechoic fluid accumulation in the vicinity of the liver or ventral abdomen immediately after biopsy	0	0
Hematoma within the liver tissue at biopsy site	Ultrasonographic visualization of dilatation of biopsy site with anechoic content immediately after biopsy	3	0.94
Delayed effects examined using clinical examination (n = 320)			
Diffuse inflammation of the skin incision site	Painful, warm swelling of the skin surrounding the stab incision, exudate, or both	42	11.9
Peritonitis	Poor appetite, arched back, abdominal guarding, fever, reduced intestinal motility. Ultrasonographic visualization of intraabdominal fluid; abdominocentesis produces exudate (Dirksen et al., 2006).	0	0
Delayed effects examined using ultrasonography (n = 240)			
Abscessation of the skin or subcutis	Ultrasonographic visualization of encapsulated mass with echogenic content in the skin or subcutis with flow phenomena and occasional reverberation artifacts and acoustic shadowing (Braun and Flückinger, 1997)	11	4.60
Cavity formation in the liver	Ultrasonographic visualization of nonencapsulated mass with anechogenic content in the liver (Herzog et al., 2004).	1	0.50
Perihepatitis	Ultrasonographic visualization of hyperechogenic deposits on the liver surface	1	0.50
Increased echogenicity of the biopsy site in the liver	Ultrasonographic visualization of a previous biopsy site in the liver characterized by hyperechogenic borders	27	10.4

scale of the trait and as a measure of variability the minimum and maximum.

The analyses of first-service conception rate and stillbirth rate were based on a binomial distribution. For the trait time to pregnancy, the evaluation was based on survival analysis. Here, we present curves (so-called failure curve in case of time to pregnancy) based on the Kaplan-Meier method, their statistical comparison (logrank test) and hazard ratios as well as their confidence intervals.

Analysis of Procedure-Related Adverse Effects. The analysis of procedure-related adverse effects was due to the binary scale of the traits based on a binomial distribution. For the complications with the greatest occurrence, "diffuse inflammation of the skin" and "increased echogenicity of the biopsy site in the liver," the influence of BCC treatment was estimated by including treatment as a categorial covariable. For the other complication this type of analysis was not performed because of the low number of individuals included. The value of the covariable for animals with BCC injection (group VER5 and VER10) is 1 (BCC occurs), for all other animals 0 (BCC does not occur). The analysis revealed P-values of the regression coefficients larger than 0.1. Therefore, this covariate was not included in the final evaluation model. The estimated probability of the occurrence of a trait per single procedure was interpreted as the complication rate.

Analysis of Milk Production Traits. Analysis of milk production traits in the study lactation was based on a fixed-regression test-day model (Mielenz et al., 2006) that considered environmental effects as fixed test-day effects. The relationship between milk yield and DIM was modeled using a second-order fractional model, and the relationship between milk fat, milk protein, and SCS and DIM with the Ali-Schaeffer model (Ali and Schaeffer, 1987). The regression coefficients were considered group specific. Both model approaches provide estimates of the group least squares means per DIM and thus lactation curves and estimates for different lactation periods. Repeated measures within cow were considered by using random animal effects and covariances of the residual effects. Here, the Toeplitz structure proved to be advantageous for all traits when testing different structures and selecting with AICC.

The influence of the BCC injection on the milk production traits was tested via a categorical covariable. The analysis revealed P-values of the regression coefficients much larger than 0.1 for all traits. Consequently, the estimates had wide confidence intervals with inclusion of zero. Therefore, this covariate was not included in the final evaluation model.

Furthermore, the influence of the performance of the pre-lactation was investigated by using a quantitative covariate. The respective regression coefficients consistently showed P-values of 0.001 and were included in the evaluation model. However, there was no need to consider group-specific regression coefficients; no significant differences between the regression coefficients of the groups could be detected.

Analysis of Fertility and Culling Data. The analyses of first service conception rate, stillbirth rate, type of culling, and reason for culling were based on a binomial distribution. Here, the influence of BCC was also investigated. Only in the case of the reason for culling "metabolic disease" was the covariable included in the final evaluation model ($P = 0.075$).

For comparison of groups, we used for all traits described above the t-test, and in the case of milk production traits for verification of normal distribution of the residuals we used the Shapiro-Wilk test.

For the traits time to pregnancy and time to culling, the evaluation was based on survival analysis as described for the pre-study lactation. The influence of BCC was also tested here. The covariate in question resulted in $P = 0.20$ for time to pregnancy and $P = 0.12$ for time to culling, and was accordingly not included in the final evaluation model.

The computational implementation was carried out with the SAS software (SAS 9.4, SAS Institute Inc.) by utilization of the procedures UNIVARIATE, MIXED, GLIMMIX, NLMIXED, IML, LIFETEST, and PHREG.

RESULTS

Adverse Effects of the Experimental Procedures in Group EXP

The clinical and ultrasonographic examinations yielded abnormal findings that were related to the experimental procedures. Liver biopsy had the highest complication rate; diffuse inflammation of the skin incision site and increased echogenicity of the biopsy site in the liver occurred in more than 10% of cows. Other complications were less common (Table 3).

Comparison of EXP and CTR Groups

Production. The lactation curves for milk yield per day (kg), protein (%), fat (%), and SCS of both groups are shown in Figure 1 and Table 4. The curves had typical shapes characterized by a rapid rise to a peak followed by a decline (kg of milk), or a decrease to a nadir followed by an increase (protein %, fat %, and SCS). The covariable previous lactation milk yield, fat %, protein %, and SCS were proved to be significant (P = 0.001) for the statistical model and were therefore included in the final model. Lower milk yields were observed in EXP cows during early (DIM 5–50, difference: 2.3 kg, SE 0.8 kg, $P = 0.01$) and late lactation (DIM 251–300, difference: 2.0 kg, SE: 1.0 kg; DIM 301–350, difference: 2.3 kg, SE: 1.2 kg; $P = 0.05$), resulting in lower 305-d milk yield in the EXP group than in the CTR group (difference: 472 kg, SE: 214 kg; $P = 0.05$). The milk fat content was higher in the EXP group than in the CTR group from 251 DIM onward (DIM 251–300, difference: 0.20%, SE: 0.09%, $P = 0.03$; DIM 301–350, difference: 0.41%, SE: 0.17%, $P = 0.02$). No difference was observed for milk protein % between groups throughout lactation, but SCS was higher in the EXP group during early lactation (5–50 DIM, difference: 0.43, SE: 0.22, $P = 0.05$) and from mid-lactation onward (DIM 151–200 difference: 0.43, SE: 0.20; DIM 201–250, difference: 0.49, SE: 0.22; DIM 251–300, difference: 0.55, SE: 0.25; DIM 301–350, difference: 0.61, SE 0.28; $P = 0.03$).

Fertility Traits. The first service conception rate did not differ between groups, with 40 ± 6.3% (LSM ± SE) in the EXP group and 38 ± 3.7% in the CTR group ($P = 0.78$). Also, time to pregnancy did not differ between groups ($P = 0.86$; Figure 2B), with a hazard ratio for CTR versus EXP of 1.14 and the respective confidence interval of 0.77 and 1.70 (2-sided confidence level $P = 0.95$). The stillbirth rate in the EXP group was lower than in the CTR group (0 ± 0% vs. 3.9 ± 1.4%; $P = 0.02$).

By comparison, the stillbirth rate of all other cows in the herd (n = 366, excluding EXP and CTR) was considerably higher than in the EXP and CTR groups

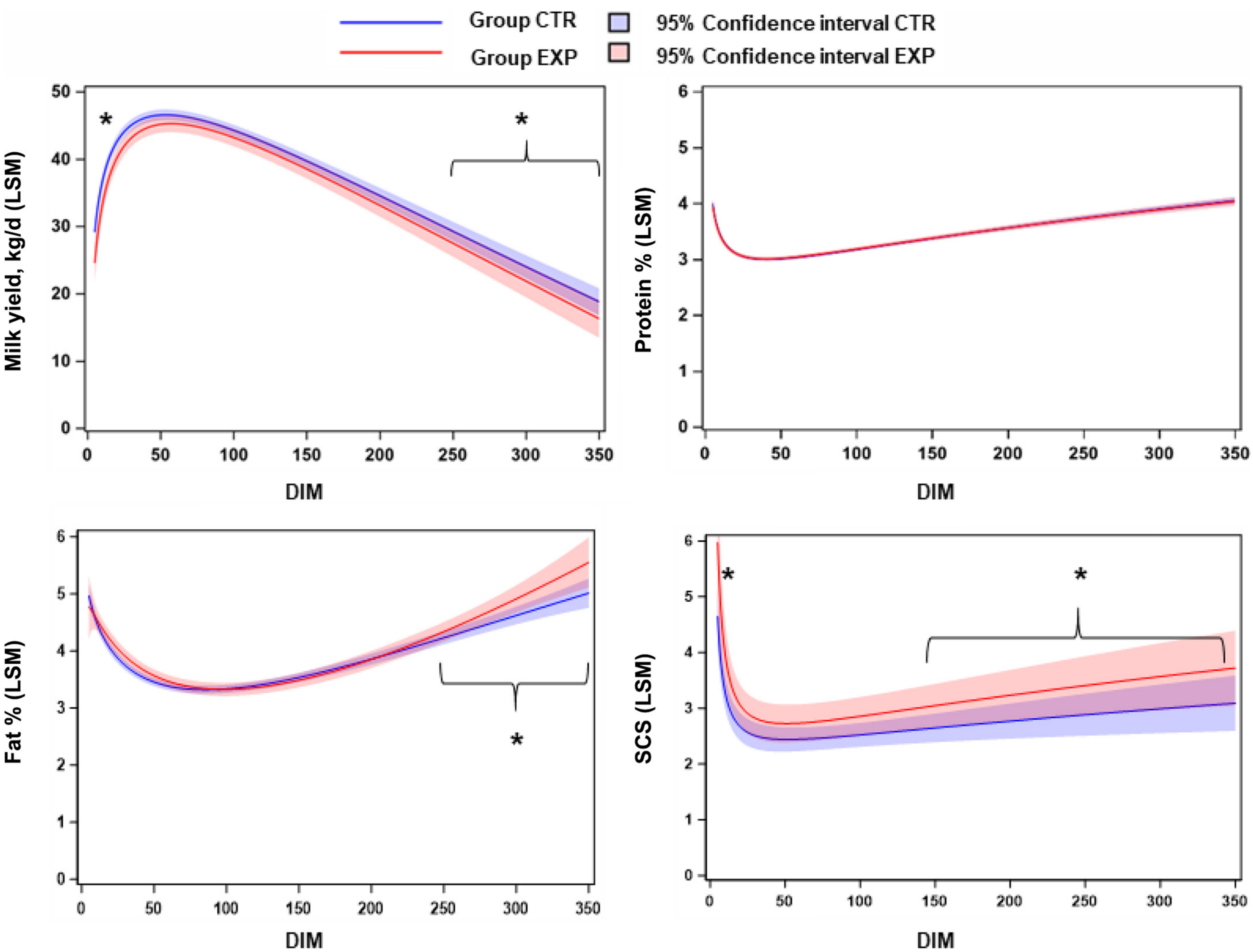

Figure 1. Production traits (milk yield in kg per day, milk fat %, milk protein %, and SCS; LSM) of cows in the experimental (EXP, n = 80) and control (CTR, n = 206) groups for the study lactation; $^*P \leq 0.05$.

during the study period and was 14.6% in 205 first-lactation cows and 10.6% in 161 multiparous cows.

Culling. No cows in the EXP and CTR groups left the herd during the study period. Four years after the end of the study, 10 cows (12.5%) in the EXP group and 14 cows (6.8%) in the CTR group remained in the herd. Time to culling was not different between groups ($P = 0.735$), the hazard ratio CTR versus EXP was estimated at 0.78, and the confidence interval was 0.55 and 1.12 (2-sided, confidence level $P = 0.95$). Details related to culling for this period are shown in Table 5 and Figure 3. Type of culling did not differ between the groups ($P > 0.05$), whereby poor production was a more frequent reason noted for culling in the EXP group ($P = 0.02$). The BCC treatment as a covariable had an influence on the culling reason "metabolic disease," with fewer cows leaving the herd due to metabolic diseases (n = 2) in the group treated with BCC than in the PLAC group (n = 5; regression coefficient = −0.591).

DISCUSSION

This study showed that an intensive experimental protocol involving serial invasive experimental sampling procedures in transition dairy cows had several direct adverse effects and negatively affected production traits. At the same time, cows in the experimental group had a lower stillbirth rate than controls.

Adverse Study Effects

Adequate restraint of the cows in the examination chute was a prerequisite for this investigation. At the start of the study, one cow sustained a leg injury in the chute and subsequently was excluded from the study. Injuries related to restraint depend on animal handling, type of fixation, and the temperament of the animal (Grandin, 1997).

Collection of blood from a jugular vein and intravenous administration of drugs are common procedures

Table 4. Production traits (milk yield, fat %, protein %, and SCS) of cows in the experimental EXP (n = 80) and control groups (CTR, n = 206) for the study lactation (LSM)

Production trait	EXP (n = 80) LSM	SE	CTR (n = 206) LSM	SE	Difference (EXP − CTR)	SE difference	*P*-value
Milk yield (kg/d)							
5–50 DIM	40.40	0.69	42.70	0.44	−2.29	0.82	0.01
51–100 DIM	44.59	0.65	45.74	0.40	−1.16	0.75	0.12
101–150 DIM	40.92	0.69	42.02	0.44	−1.10	0.73	0.13
151–200 DIM	35.80	0.78	37.10	0.53	−1.30	0.75	0.09
201–250 DIM	30.26	0.93	31.86	0.65	−1.60	0.85	0.06
251–300 DIM	24.63	1.12	26.58	0.80	−1.95	1.00	0.05
301–350 DIM	19.01	1.33	21.34	0.95	−2.32	1.20	0.05
305-d milk yield	10,776.00	213.70	11,248.00	138.10	−471.80	213.80	0.03
Milk fat (%)							
5–50 DIM	4.04	0.06	3.93	0.04	0.12	0.07	0.10
51–100 DIM	3.40	0.06	3.35	0.04	0.04	0.06	0.49
101–150 DIM	3.39	0.06	3.42	0.04	−0.04	0.06	0.57
151–200 DIM	3.66	0.07	3.70	0.04	−0.03	0.07	0.64
201–250 DIM	4.09	0.08	4.05	0.05	0.05	0.08	0.56
251–300 DIM	4.63	0.10	4.43	0.07	0.20	0.09	0.03
301–350 DIM	5.23	0.17	4.82	0.10	0.41	0.17	0.02
Milk protein (%)							
5–50 DIM	3.15	0.02	3.15	0.01	0.00	0.03	0.97
51–100 DIM	3.09	0.02	3.09	0.01	0.01	0.02	0.70
101–150 DIM	3.28	0.02	3.28	0.01	0.01	0.02	0.79
151–200 DIM	3.47	0.03	3.47	0.02	0.00	0.02	0.93
201–250 DIM	3.65	0.03	3.65	0.02	0.00	0.03	0.93
251–300 DIM	3.81	0.03	3.82	0.02	−0.01	0.03	0.84
301–350 DIM	3.96	0.04	3.97	0.03	−0.01	0.04	0.77
SCS							
5–50 DIM	3.18	0.18	2.75	0.12	0.43	0.22	0.05
51–100 DIM	2.78	0.17	2.47	0.11	0.31	0.20	0.12
101–150 DIM	2.95	0.19	2.59	0.12	0.37	0.19	0.06
151–200 DIM	3.14	0.21	2.71	0.15	0.43	0.20	0.03
201–250 DIM	3.32	0.25	2.83	0.18	0.49	0.22	0.03
251–300 DIM	3.49	0.29	2.94	0.21	0.55	0.25	0.03
301–350 DIM	3.65	0.32	3.04	0.24	0.61	0.28	0.03

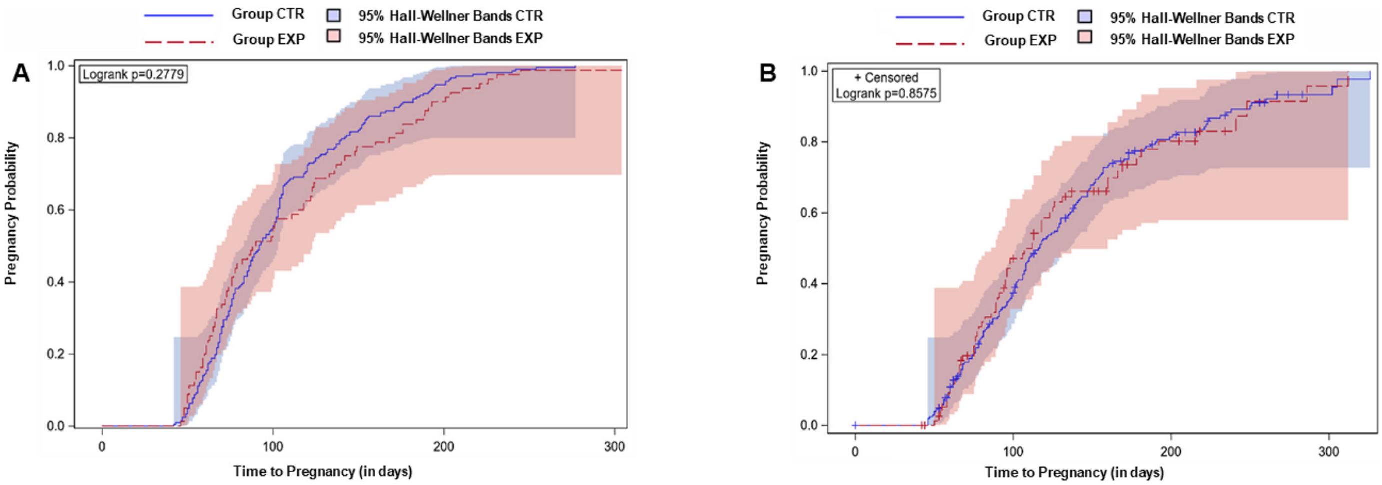

Figure 2. Time to pregnancy (d) of cows in the experimental (EXP, n = 80) and control (CTR, n = 206) groups for the pre-study lactation (A) and study lactation (B), showing no difference between groups [hazard ratios CTR vs. EXP: 1.15 and 1.14, and CI (2-sided, confidence level $P = 0.95$): 0.89; 1.50 and 0.77; 1.70, for (A) and (B), respectively].

in cattle experiments and during veterinary treatments. Venipuncture is associated with potential complications including hematoma, phlebitis, periphlebitis, and thrombophlebitis (Dirksen et al., 2006). Poor restraint, inadequate preparation of the overlying skin (Dirksen et al., 2006), and lack of operator experience (Tagalakis et al., 2002; Rouleau et al., 2003) are risk factors for thrombophlebitis. Published research on the complication rate of venipuncture without the use of an indwelling catheter is scant (literature search: PubMed, April 27, 2021, key words: *cattle*, *venipuncture*, *thrombophlebitis*). In a study in which indwelling jugular catheters were placed in 50 cows, 3 developed thrombophlebitis (Rouleau et al., 2003).

Transcutaneous liver biopsy, which was carried out 4 times in each cow, is a highly invasive procedure. The most common complications seen in the present study were diffuse inflammation of the skin incision site, abscessation of the skin and subcutis, and an increase in echogenicity of the biopsy site in the liver. In humans hemorrhage appears to be the most common complication of percutaneous liver biopsy and its incidence depends on the type of biopsy needle used and on operator experience (Friedman, 2004). Reports of complications of liver biopsy in cattle are rare; one study described skin swellings in 14 of 33 heifers, and a subcutaneous abscess in one heifer 7 d after liver biopsy (Rogers et al., 2001). Other investigators also reported inflammation of the puncture site in the abdominal wall, which they attributed, at least in part, to the suture material used to close the skin (Oxender et al., 1971). Some of the adverse effects of liver biopsy determined in our study have not been reported in cattle yet. In human medicine, hemorrhage and hematomas involving the biopsy site in the liver have been reported in up to 8% of cases after transcutaneous liver biopsy (Spinzi et al., 1988). To our knowledge, changes in the echogenicity of the liver tissue surrounding the biopsy site have not been reported in human or veterinary medicine (literature search: PubMed, April 24, 2021, key words:

Table 5. Probabilities (%) for types and reasons of culling of cows of the experimental (EXP) and control (CTR) group and their differences (EXP − CTR) for 4 yr after the end of the study

Item	EXP (n = 70)	SE	CTR (n = 193)	SE	Difference (EXP − CTR)	*P*-value (*t*-test)
Type of culling						
Slaughter	87.0	4.05	79.1	2.94	7.90	0.15
Death	1.45	1.44	6.81	1.82	−5.36	0.12
Euthanasia	11.6	3.85	14.1	2.52	−2.50	0.60
Reason for culling						
Mastitis	17.4	4.56	23.0	3.05	−5.60	0.33
Lameness	17.4	4.56	16.7	2.70	0.70	0.90
Production	15.9	4.40	6.28	1.76	9.70	0.02
Infertility	10.1	3.64	12.0	2.36	−1.90	0.67
Metabolic disease	22.2	6.90	13.6	2.40	19.8	0.19

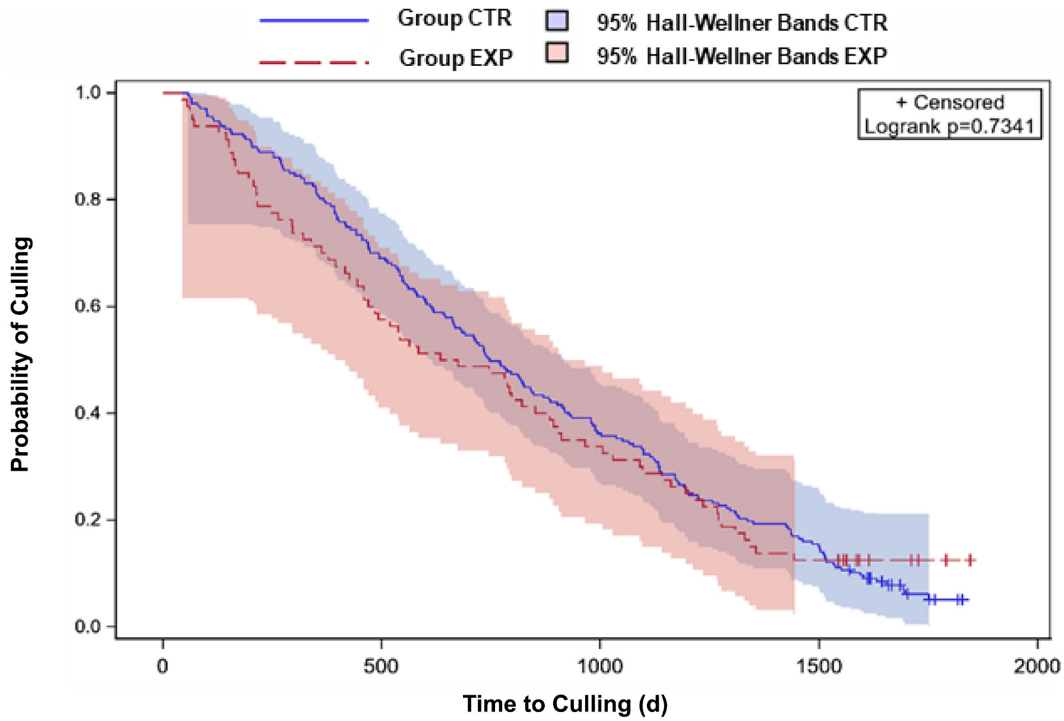

Figure 3. Time to culling (d) of cows in the experimental (EXP, n = 80) and control (CTR, n = 206) groups for the study lactation with no difference between groups [hazard ratio CTR vs. EXP: 0.78 and CI (2-sided, confidence level $P = 0.95$): 0.55; 1.12].

liver biopsy, fibrosis). Although change in echogenicity after biopsy is not itself an adverse effect we decided to document this observation, as it appeared not to be present after each biopsy procedure. The relevance of our observation is not clear to date. Liver biopsy causes mechanical trauma to the tissue, but healing occurs within a few weeks due to the regenerative capacity of the liver (Kampmann et al., 1980). After repeated liver biopsy sampling, we did not see histological changes of the liver parenchyma (Pietsch et al., 2021). An increase in echogenicity of the liver tissue was reported to be consistent with fibrosis, which can be a normal process of wound healing (Sarrazy et al., 2011). If this process is also responsible for the increased echogenicity observed here cannot be finally concluded. However, increased echogenicity of the liver biopsy site was not seen in all cows, which suggests that some cows had more pronounced reactions than others, or that healing was delayed in some. It is conceivable that pre-existing liver lesions affect healing. For example, fibrosis is a common complication of liver biopsy in humans with fatty liver syndrome, further compromising liver function (Fassio et al., 2004). Mice with fatty liver syndrome had increased expression of factors that support migration of myofibroblasts to liver lesions (Foglia et al., 2019). In the present study, liver lipid content was highest at 7 DIM (Pietsch et al., 2021), which may have compromised wound healing in some cases. Inadvertent puncture of another organ was rare and only occurred in one case. In a similar study, in which 31 steers underwent liver biopsy, intestinal content was observed in one sample but liver biopsy was completed in a subsequent attempt (Rogers et al., 2001). More serious complications such as severe hemorrhage, diffuse peritonitis, or death did not occur in the present study. The low complication rate of liver biopsy in the present study reflects optimal technique carried out by well-trained operators.

Approximately half of all cows had at least one adverse effect associated with the experimental procedures. Adverse study effects can be minimized when experienced investigators are performing the procedures (Westheim et al., 2013). In the present study all biopsy procedures were taken by a diplomate of the European College of Bovine Health Management with more than 20 yr of experience in this particular procedure. All other procedures were performed by well-trained postgraduate staff, and therefore a lack of experience can be excluded as explanation for observed adverse events. For future studies, accustoming the cows to the handling and fixation before the study should be considered as a possible risk-reducing measure for adverse effects, as studies have shown that a stress response and thereby defensive behavior can be reduced by training and gentle handling of cows (Waiblinger et al., 2004).

Whether all the reported biopsy and puncture-related adverse effects cause pain is not clear, but inflammatory processes (inflammation of the skin incision, abscessation, perihepatitis) are assumed to be painful (Zhang and An, 2007). Venipuncture and biopsy cause direct pain (Herndon et al., 1984; Goodenough et al., 1997; Castera et al., 1999) and stress because they involve handling (Mitchell et al., 1988; Frese et al., 2016), separation from the herd (Boissy and Le Neindre, 1997; Apple et al., 2005), and restraint in a chute (Andrade et al., 2001; Lindahl et al., 2016; Heinrich et al., 2020), which alter the daily routine, rest, and eating periods (Cooper et al., 2008). Although stress and pain levels were not quantified by means of behavioral analysis (Ede et al., 2019) or feed intake, it is very likely that the experimental procedures had an effect on these traits.

Effects on Production

Stress, pain, and changes in daily routine result in direct changes of the physiological state such as reduction in feed intake or immunosuppression with effects on production (Moberg, 2000).

Milk Yield. Handling and procedures such as foot trimming, liver biopsy, or temporarily separating dairy cows from pen mates causes a transient decrease in milk yield without affecting the milk components (Rushen et al., 2001; Cooper et al., 2008; Khatun et al., 2013). This was confirmed in our study even though other investigators did not observe an adverse effect of liver biopsy on milk yield (Vels et al., 2009; Weng et al., 2017). Decreased milk yield may have been the result of lower feed intake, higher energy demand, or stress-related activation of the hypothalamic-pituitary-adrenal axis. Corticosteroids reduce milk production (van der Kolk, 1990; Ollier et al., 2016) possibly by inducing changes in the tight-junction permeability of the mammary epithelium (Stelwagen et al., 1998) or by reducing mammary glucose uptake (Hartmann and Kronfeld, 1973). The lower production in the EXP group in the first 2 mo of lactation may have been due to the combined effects of experimental stressors and hormonal and metabolic changes (Huzzey et al., 2011) because of overlap of the study period with the transition period.

Besides the lower milk yield, the cows of the EXP group had a higher SCS than cows in the CTR group throughout lactation, particularly in the beginning and end of lactation. Other studies described similar increases in SCC in response to various stressors (Wegner et al., 1976; Yagi et al., 2004). In a study similar to ours, in which liver and udder biopsies were done, an increase in milk SCC was paralleled by leukocytosis, but this was monitored for only 10 d (Khatun et al., 2013). The increase in milk SCC in the EXP group may be attributed to stress-induced activation of the hypothalamic-pituitary-adrenal axis, resulting in immunomodulation (Moberg, 2000), which may manifest as leukocytosis in the mammary gland (Wegner et al., 1976). We furthermore suggest that the differences in SCS between groups in late lactation were related to the lower production of the animals in the EXP group toward the end of lactation because reduced production in late lactation has been associated with an increase in SCC (Hagnestam-Nielsen et al., 2009).

For the interpretation of the milk yield data, it must be considered that values were extracted from a monthly milk control report (with only 1 or 2 measurements per animal during the experimental period), where possibly not all study effects on milk production are depicted.

Fertility Traits. With the exception of stillbirth rate, our experimental procedures did not affect fertility traits. Similarly in other studies, different restraint methods and low-stress handling during fixed-time AI had no effect on pregnancy rates (Tirloni et al., 2013; Carrell et al., 2021). Huzzey et al. (2015) however showed a negative correlation with the concentration of stress-associated fecal glucocorticoid metabolites (11,17-dioxoandrostane) of transition cows and their later conception rate.

Interestingly, the experimental procedures had a positive effect on stillbirth rate, which was lower in cows of the EXP group than in cows of the CTR group. We are not aware of other studies investigating the effect of experimental procedures on stillbirth rate in cattle but stress is generally believed to increase stillbirth rate in a variety of species (Wiebold et al., 1986; Beydoun and Saftlas, 2008; Wisborg et al., 2008). The prepartum study period of 14 d may have been too short, and the manipulations too mild to have an adverse effect. The stillbirth rate was considerably larger in the remaining herd (excluding EXP and CTR groups), which may have been related to preselection of the cows used in the study. Inclusion criteria favored healthy multiparous cows, whereas the remaining herd had relatively more primiparous and pretreated cows and possibly more cows with diseases. Lactation number and health status have a significant effect on stillbirth rate (Mee, 2008). Another factor favoring the birth of live calves was close monitoring of cows in the EXP group by the veterinary staff conducting the study. Close monitoring of calving cows (Hodge et al., 1982), video surveillance of the calving pen (Vernooy et al., 2007), and training of calving pen attendants (Kausch, 2009) all contribute to a lower stillbirth rate. However, it must be considered that the multiparous cows of the EXP group had numerical, although not statistically significant, lower stillbirth rates than the CTR group in the previous calving cycle.

Culling. Despite the observed complications, no cows of the EXP group left the herd within 42 DIM, and the 2 groups did not differ with respect to cull rate, time to culling, or reasons for leaving the herd in the study lactation. By 48 mo after the end of the study, about 90% of cows in both groups had left the herd, which corresponded to a herd turnover rate of 25 to 30% (Fetrow et al., 2006). Most cull cows left the herd due to disease (lameness, metabolic disease, mastitis, or infertility) or low production, and the latter reason was more common in the EXP group than in the CTR group, which is in accordance with the lower 305-d milk yield of cows in the EXP group. Reliable records of disease incidences during the study period were not available for the CTR cows and therefore between-group comparisons could not be made. Collection and reporting of data related to the health of cattle under production conditions are difficult (Kelton et al., 1998). Furthermore, BCC treatment had an influence on the culling reason "metabolic disease." The BCC has been reported to decrease the prevalence of subclinical ketosis (Rollin et al., 2010); however, it has to be considered that the reason for culling was documented by the herd manager, and hence these diagnoses need to be interpreted with caution.

General Discussion and Limitations

Intensive experimental protocols have measurable effects on dairy cows. A comparison of our findings with those of other studies is difficult. The number of research projects involving dairy cows is considerable, but in most research institutions it is not possible to include large control groups that are kept under the same production conditions and not handled. On the other hand, many field studies do not involve experimental procedures as intensive as those carried out in this study. Another reason why comparison with other studies is difficult is that there is not always a designated space in a publication for the report of adverse effects and production losses caused by experimental procedures. However, in the last few years, several scientific journals have started to adopt reporting guidelines on aspects of study design and animal experimentation, that include the reporting of adverse outcomes (e.g., item 9 in the REFLECT statement reporting guidelines, Sargeant et al., 2010).

When drawing conclusions from our study, several limitations have to be accounted for. The effects documented during the study are most likely the result of combined and additive effects of the different applied procedures, and an extrapolation to other studies using less or different experimental measures might be limited. Furthermore, the study was performed on only one farm with one experimental protocol, limiting its extrapolation to other cohorts.

Different aspects during the analysis of this trial have however led us to the conclusion that future trials with such intensive protocols should include an additional treatment group (in our case for butaphosphan and cyanocobalamin) that is not manipulated for further examination and sample collection, and only production data (milk yield and components, and BCS) are recorded to anticipate possible confounding factors (discussed in Schären et al., 2021a,b).

It is crucial that experimental studies involving dairy cows are planned and supervised by qualified researchers and examinations and procurement of samples are conducted by well-trained staff to avoid or minimize potential adverse effects. Optimum working conditions at the site of the experiment contribute to a successful outcome. Potential production losses must be considered when planning a study and possible compensation claims should be arranged with the owner. At the same time, the herd owner should be made aware of the positive aspects of a research study in a commercial herd; experimental cows are carefully monitored and receive treatment when necessary. Furthermore, it facilitates knowledge sharing between veterinarians and staff, and infrastructure components including examination equipment and laboratory installation may remain available after the completion of the study.

CONCLUSIONS

Our study illustrates that intensive experimental procedures undertaken in transition dairy cows can negatively affect health and production. At the same time positive effects on calving-related traits, possibly caused by an intensive monitoring of the experimental animals, were observed. The negative and positive aspects of experimental procedures should be considered in the planning phase of a study. This analysis of procedure-related impairment of production in dairy cows is unique and allows the estimation of the effect of such an intensive study protocol on animal welfare and production. As in our study only a limited number of animals were included on one single farm, the extrapolation of the results is limited. It is encouraged that similar analyses are conducted retrospectively on data of previous studies, as well as specifically included in the design of future studies.

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