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Evaluation of an investigative model in dairy herds with high calf perinatal mortality rates in Switzerland

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Short title: Perinatal mortality on high risk dairy farms in Switzerland

Abbreviations:

PM	Perinatal mortality
PP	Primiparous
MP	Multiparous

MAT	Microscopic agglutination test
qPCR	Real-time PCR
PL	Placenta or foetal fluids
AC	Abomasal contents
AFC	Age at first calving
COD	Cause of death
UCOD	Ultimate cause of death
PCOD	Proximate cause of death
TOD	Time of death
IC	Inbreeding coefficient

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Abstract

The objective of this study was to evaluate an investigative model which encompassed the risk factors, incidence, timing and causes of perinatal mortality (PM) (0 to 48h) on high risk dairy farms (PM of >5% in the previous year) in Switzerland. This pilot-study was carried out on 47 predominantly Holstein PM calves from 21 dairy farms, between September 2016 and January 2018.

Gross pathological examinations of calves and placentae as well as histopathological examinations of internal organs and placental tissue were performed. Further investigations included microbiological examinations: broad-spectrum bacterial and fungal culture, detection of *Chlamydia abortus*, *Coxiella burnetii*, pathogenic *Leptospira* spp. and *Neospora caninum* by real-time PCR (qPCR) and of bovine viral diarrhoea virus (BVDV) by Ag-ELISA. Maternal blood samples were used for serology of bovine herpesvirus 1 (BHV-1), *Brucella abortus, Chlamydia abortus, Coxiella burnetii* and nine pathogenic leptospiral serovars and the evaluation of trace element status. A questionnaire was completed with the farmer, which included general farm characteristics and case-related data. Inbreeding coefficients (IC) were calculated for pure-bred matings.

At the farm-level, the PM rate was 10.0% (5.3-28.2%) and at the cow-level, 11.5%. These values, from high-risk farms, were approximately five-times higher than the contemporary national bovine PM rate (2.3%) in Switzerland. The risk factors associated with these high PM rates were the self-selection of high risk herds, the high proportion of primiparae in these herds (45%) and the evidence of widespread pathogenic infections on these farms (exposure: 67% of herds, 53% of dams; infection: 57% of herds, 45% of calves). The majority (68.1%) of calves died intrapartum. The most commonly diagnosed initiating/ultimate cause of death (UCOD) was infection (34%) of which *Coxiella burnetii* was the most frequently detected pathogen, by antigen. The most frequently diagnosed proximate cause of death (PCOD) was asphyxia (44.7%), though multiple PCOD was also common (21.3%). This study was the first detailed investigation of bovine PM in Switzerland. Infectious causes were diagnosed more frequently than expected. While the findings from these high PM Swiss herds may have limited external validity, the investigative model adopted and the detailed research

methodologies employed can be replicated and re-evaluated, respectively, in future studies on PM internationally.

Keywords: perinatal mortality, stillborn calves, infection, *Coxiella burnetii,* necropsy, Switzerland

1. Introduction

High calf mortality rates cause high economic and genetic loss and increased costs for replacements [1]. Bovine perinatal mortality (PM) is commonly defined as the mortality of full-term (≥ 260 days pregnancy) calves shortly before, during or up to 24 to 48 hours after parturition. This case definition varies among studies [2,3]. Stillbirth is often used as a synonym for PM, but should correctly be defined as the death of a foetus before or during calving at full-term, not including death in the period after calving [4]. A PM rate >5% (per year and herd) is used by the majority of European veterinarians as the investigation threshold [4,5]. The highest mortality occurs within the first hours of life [1,6] and incidences are higher for primiparous (PP) than for multiparous (MP) cows [7].

In Switzerland, Bleul [6] described a PM rate of 2.4% during the first 24 hours (2005 to 2007). This value is much lower than those described in most other countries (e.g. USA: 7.0 to 8.8%, Germany: 9.3 to 9.7%) [8–11].

Mee et al. [12] differentiated between modifiable [age at first calving (AFC), breed, breeding method, calving management, foetal sex, foeto-maternal health status, gestation length, gestational nutrition, sire] and non-modifiable risk factors (parity, history of previous PM, twins, foetal presentation, herd, herd size, calving day of the week, season and year). Modifiable risk factors can be improved by farm management, whereas non-modifiable factors cannot or can only minimally be influenced by the farmer. Most authors agree that dystocia is the main reason for a high PM rate [3,1,6,13,14]. Dystocia also increases the calves' stress, reduces the transfer of passive immunity through delayed colostrum intake and, therefore, leads to higher postnatal morbidity and mortality [14]. Calves born to dams younger than two years of age are at increased risk for PM [6,13], and calves born before 272 days or after 302 days of gestation have a higher risk of PM than those delivered between 272 and 302 days [6]. Male calves as well as twins and calves born in the colder months are also at higher risk than females, singletons and calves born in spring or summer, respectively [3,6,13]. Infectious causes of PM include *Neospora caninum*, *Salmonella* spp., *Leptospira* spp., *Coxiella burnetii*, *Brucella abortus*, *Chlamydia abortus*, *Listeria* spp., *Trueperella pyogenes*, different ubiquitous and opportunistic fungi such as *Aspergillus* spp., bovine viral diarrhoea virus (BVDV) and bovine herpesvirus 1 (BHV-1) [4,15]. It was speculated

that the high calf mortality rates in American Holsteins [16] and Red Holsteins in Switzerland might be due to a common genetic cause [6]. Improper nutrition before parturition including inadequate dietary energy, fat, protein and trace element supply (iodine, selenium, copper, zinc) is described as a risk factor for a high prevalence of PM [12,13].

In order to provide a guide to structured investigation of abortion/stillbirth/PM cases, Mee [17] established an investigative model using standard operating procedures (SOPs) for practitioners. Three steps are involved in the model: collecting a history, examination of pregnant animals and animals suffering from abortion/stillbirth, as well as examination of the dead calf and the placenta [4,17]. Examining pregnant animals and their environment allows an overview of their general health, body condition score (BCS) and feeding management [4].

In Switzerland, farmers and practicing veterinarians perceive an increasing rate of PM (personal experience). However, only one study was carried out in Switzerland on PM, over a decade ago, which reported an increasing PM rate from 1.8% to 2.8% (2005-2007) [6]. The study neither examined calves nor their dams nor were any further investigations undertaken [6]. Therefore, given the paucity of recent information on PM in Swiss herds and the absence of information on the associated epidemiology and pathology, the present study was conducted to fill these knowledge gaps. The research study afforded an opportunity to evaluate an investigative model by collecting epidemiological information and dead calves, placentae and maternal blood from herds with high PM risk for necropsy and laboratory analyses.

2. Materials and Methods

This investigative model involved: (1) recruitment of high PM risk herds, (2) submission of samples (calves, placentae and amniotic fluid) from these herds by farmers, (3) visit to these farms to collect samples (blood, milk), examination of the dam and interview the farmers based on a questionnaire (4) necropsy and laboratory examination of the samples collected, (5) retrieval of calf mortality records for the recruited herds from the national database (http://www.agate.ch) and (6) retrieval of national-level calf mortality records from the national database.

2.1. Study population

The herd-level inclusion criterion as a high-risk farm was a Swiss herd with an (elevated) PM rate (>5%) in the twelve months before referring the first calf. To recruit farms, a summary of the project was distributed to the Swiss dairy farmers through the breeding associations, and a short description of the project was presented in a nationwide continuing education programme for bovine practitioners in Bern in September 2016. The animal-level inclusion criterion was a calf born after ≥ 260 days of pregnancy, which died shortly before or during parturition or within the first 48 hours of life. Between September 2016 and January 2018, we received 50 calves, 42 corresponding placentae and 4 samples of amniotic fluid from 48 dams, originating from 23 herds. In two cases (no. 28 and 39), neither placenta nor amniotic fluid was available. Due to different management factors between dairy and beef herds, two beef calves (case no. 30 and 34) and their farms (farm no. 17 and 20) were excluded from the study. A third case (no. 45) was excluded to reduce the bias within the same dam (PM calves in two subsequent calving seasons). In the end, 21 farms, 47 calves, 45 corresponding dams, 40 corresponding placentae and 3 samples of amniotic fluid were included in the statistical analysis. Ethical authorization of this study was granted following notification of the local veterinary authorities as an animal experiment grade zero (BE 73/16) for additional blood and milk sampling.

2.2. Blood sampling

Within a median (min, max) of six days (0 days, 23 days) after parturition, two coccygeal blood samples [S-Monovette® (no anticoagulant) Luer tubes, Sarstedt AG, Nümbrecht, Germany] were collected from all dams. The blood samples were centrifuged within two hours of collection at 3,500 revolutions per minute for 10 minutes (Hettich® EBA 20 centrifuge, Hettich AG, Switzerland) and the sera stored in Eppendorf tubes. One tube was sent directly to the Institute of Bacteriology (Vetsuisse Faculty, University of Bern, Switzerland) for serological analyses, one tube was sent to the Idexx Diavet laboratory (Bäch, Switzerland) for BHV-1 serology and two samples were stored at -20 °C for trace element analysis.

2.3. Examination of the dam

The dam was examined at the time of blood sampling and the body condition was scored from 1 to 5 using the Pennsylvania method [18]. The pelvic angle was scored by linear description as straight, downwards or upwards according to the national guidelines [19]. The udder filling at the time of calving was evaluated by the farmer as good or poor.

2.4. Questionnaire

A questionnaire was completed during a face-to-face interview with the farmer during the farm visit. The questionnaire was divided into two parts. In the first part, which was completed only once after the first calf loss, information on the general farm characteristics was collected: herd size, cow husbandry (tie or free stall), main breed, type of income (main income or additional work), number of external staff, label production (e.g. organic, IP-Suisse), other animal species, rearing/cattle trade, feeding, mineral supplementation, calf husbandry, newborn and colostrum management, dam vaccination, general calving management, type and storage of calving aids, cleaning and disinfection in calving and calf's area. The second part of the questionnaire comprised case-related data and was administered separately for each individual case. The following information was collected on the dam: ear tag number, breed, parity group (PP or MP), calving age, insemination technique (including ear tag number and breed of

sire), usage of sexed semen, gestation length, dry period length, calving induction, time of discharge of foetal membranes, calving time [day (6 am - < 6 pm) or night (6 pm - < 6 am)], observation of amnion sac and calving itself, timing of calving assistance after onset (defined as expulsion of amnion sac or visible legs), calving aid (none, 1 person, > 1 person and/or veterinarian), clinical dystocia, reasons of dystocia, health status of the dam (healthy or sick, regardless of the reason) before and after calving, previous dystocia of the respective dam and changes on farm (purchases, stable change, staff change) in the last three months prior PM. The following information was collected on the calf: breed, gender, number of calves, colostrum intake (yes or no), agonal clinical signs and details of death. The farmer's impression of the time of death (TOD) was determined in two categories as stillbirth vs. no stillbirth.

2.5. Pathological examination of calves and placentae

2.5.1. Calves (n = 47), placenta (n = 40) and amniotic fluid (n = 3) were transported by the farmers within 2 days after calf death to the Institute of Animal Pathology (Vetsuisse Faculty, University of Bern, Switzerland) for further investigations. Necropsy examinations were performed on all calves at the Institute of Animal Pathology (Vetsuisse Faculty, University of Bern in Switzerland) within 48 hours after parturition (stillbirth) or death (PM) using the SOP designed by Mee [17]. The calf weight and crown-rump length (distance between the external occipital protuberance and the end of the sacral bone measured on a straight vertebral column using a measuring tape; in case of rigor mortis the contour of the spine was followed) was recorded. Both the calves and their placentae were examined visually before dissection of the calves and internal visual examination. The placental weight and number and size of cotyledons (minimal and maximal diameter) were recorded. The thyroid gland was entirely removed from the underlying tissue, and its weight was measured on a precision balance (Mettler-Toledo Viper SW L0106-GDSc01, Mettler-Toledo GmbH, Switzerland). For microbiological analyses, an ear-notch sample, the ligated abomasum, one lung, one liver lobe, one brain hemisphere and placental cotyledonary tissue were collected. For histopathological examination, samples of lung, liver, kidney, thyroid, myocardium, laryngeal musculature, the other brain hemisphere of the calves, and cotyledonary and

intercotyledonary placental tissue (adjacent to areas sampled for microbiological analyses) were collected and fixed in 10% formaldehyde for 24 to 48 hours. These tissue samples were paraffin-embedded and processed to haematoxylin and eosin-stained tissue sections. In case of a positive result for *Neospora caninum* by real-time PCR (qPCR) additional sections from the brain (cerebral cortex, thalamus, brainstem and cerebellum) were processed. For all other cases, no histology was performed from the brain samples. In one case, a mummified foetus, no samples were collected for histopathology. The histopathological tissue sections were evaluated by one board-certified pathologist for the presence of inflammatory and degenerative lesions.

2.6. Microbiological examinations

2.6.1. Broad-spectrum bacterial and fungal culture

Broad-spectrum bacterial and fungal culture was performed according to Schnydrig *et al*. [20] for all samples of placenta (PL), abomasal contents (AC), liver and lung. In 4 cases the placenta was not available and amniotic fluid was examined instead.

2.6.2. Total DNA extraction

Extraction of total genomic DNA was performed for all samples of PL and AC with the KingFisher™ Cell and Tissue DNA Kit on the KingFisher™ Duo Prime Purification System (ThermoFisher Scientific Oy, Vantaa, Finland). For placental tissue, a piece of approximately 2 cm diameter of a cotyledon was homogenized in 5 ml of 0.85% NaCl using an Ultra-Turrax® Tube Drive Workstation (IKA®-Werke GmbH, Staufen, Germany). Extraction was carried out according to the manufacturer´s protocol using 200 µl of the homogenate of the placenta and 200 µl of the AC.

2.6.3. Molecular detection of *Chlamydia abortus*, *Coxiella burnetii*, pathogenic *Leptospira* spp. and *Neospora caninum*

The qPCR targeting the *ompA* gene of *Chlamydia abortus* was performed with primers and probe and cycling conditions according to Pantchev et al. [21] on an Applied Biosystems® 7500 Fast qPCR System using 1x TaqMan® Fast Advanced Master Mix, 0.5x of internal positive control (IPC) template, 0.5x IPC Mix (Applied Biosystems,

Foster City, CA, USA) and 2.5 µL of the template. The qPCRs targeting IS*1111* of *Coxiella burnetii* and *lipL*32 of pathogenic *Leptospira* spp. was performed according to Vidal *et al*. [22]. For molecular detection of *Neospora caninum* the foetal brain was sent to the veterinary diagnostic service of the Institute of Veterinary Parasitology, Vetsuisse Faculty, University of Bern, Switzerland. Total DNA was extracted as described in Schnydrig et al. [20] and the qPCR targeting Nc5 of *Neospora caninum* was performed according to Müller et al. [23].

2.6.4. Bovine viral diarrhoea virus

Enzyme-linked immunosorbent assay (ELISA) for BVDV antigen detection in ear-notch samples was performed at the Idexx Diavet laboratory (Bäch, Switzerland) with the IDEXX BVDV Ag/Serum Plus (IDEXX Switzerland AG, Liebefeld-Bern, Switzerland) according to Hilbe et al. [24].

2.6.5. Serology

The sera from the dams were tested for antibodies against *Brucella abortus*, *Chlamydia abortus* and *Coxiella burnetii* using the commercial ELISA test kits IDEXX Brucellosis Serum X2, IDEXX Chlamydiosis Total Ab and IDEXX Q Fever, respectively (IDEXX, Liebefeld-Bern, Switzerland) according to the manufacturer's instructions at the Institute of Bacteriology, Vetsuisse Faculty, University of Bern, Switzerland. The results derived from the ratio between optical density (OD) of the sample (S) and the OD of positive control (P) included in the kits and was expressed as S/P values. For the IDEXX Brucellosis Serum X2 test the interpretation was as follows: S/P < 70% was considered negative, S/P ≥ 80% was considered positive and S/P values in between were considered suspect positive. For the IDEXX Chlamydiosis Total Ab and Q fever, an S/P ≥ 40% was considered positive, an S/P < 30% was considered negative, and S/P values in between were considered suspect positive. The serological detection of antibodies against *Leptospira* spp. was performed by microscopic agglutination test (MAT) [25]. The following strains were used to detect antibodies against nine pathogenic leptospiral serovars (*L. kirschneri* serovar Grippotyphosa strain Moskva V; *L. borgpetersenii* serovars Ballum strain Mus127; Sejroe strain M84 and Tarassovi strain Perepelitsin; *L.*

interrogans serovar Australis strain Ballico; Pomona strain Pomona; Canicola strain Hond Utrecht IV; Icterohaemorrhagiae strain RGA and Hardjo strain Hardjoprajitno). Sera were screened initially for agglutination at a dilution of 1:100. Reactive sera were then titrated in two-fold serial dilutions to determine the end-point titre defined as the dilution at which at least 50% agglutination occurred. BHV-1 antibodies were detected using the IDEXX IBR gB X3 Ab ELISA (IDEXX Switzerland AG, Liebefeld-Bern, Switzerland) according to the national guidelines [26,27].

2.6.6. Follow up *Coxiella burnetii* and *Leptospira* spp. investigations

In the case of a positive qPCR result and a negative or suspect positive serology result for either *Coxiella burnetii* or *Leptospira* spp., a follow-up investigation was performed. Three to four weeks after the first blood sample collection, a second blood sample, and in the case of *Coxiella burnetii,* an additional sterile four-quarter milk sample, was collected. Both sample types were again submitted to the Institute of Bacteriology (Vetsuisse Faculty, University of Bern, Switzerland). The blood samples were subjected to serological analyses as described above. From the milk samples, DNA was extracted according to Rossetti et al. [28], and the *Coxiella burnetii* qPCR was performed as described [22]. There were seven and three follow-up investigations for coxiellosis and leptospirosis, respectively.

2.7. Determination of the time of death (TOD)

The time of death of the calves was classified into three categories: prepartum, intrapartum or postpartum. The TOD was determined by the degree of pulmonary atelectasis, the degree of autolysis [using the pathologist's description of carcass quality (no, slight, moderate or severe autolysis)] and evidence of postnatal survival (farmer's impression of TOD) [7].

2.8. Determination of the cause/s of death (COD)

The cause of death (COD) was investigated in two different ways. Firstly, the ultimate cause of death (UCOD) answered the question '*why* did the calf die?', and secondly the proximate COD (PCOD) answered a subtly different question '*how* did the calf die?' The

former is the question most commonly asked by farmers and their veterinarians while the latter is asked by veterinary pathologists. The UCOD may be defined as the single, initiating, underlying, root cause in a chain of events leading to the calf's death, in the absence of which the calf would not die. The PCOD may be defined as the terminal, immediate, fatal event or pathological mechanism/mode of death in the causal sequence initiated by the UCOD (Mee, in press). For each case, a UCOD and a PCOD was assigned using the clinico-pathological evidence from the case history by the farmer, the necropsy examination and the laboratory investigations. Given the limited number of cases investigated, the UCOD was categorised into two groups based on the most commonly diagnosed category (infection); infection and non-infection for statistical analysis. Non-infectious causes included dystocia, premature placental separation, multiple COD (co-mortalities where more than one COD was identified) and diagnosis not reached (DNR). For the same reason the PCOD was categorised into two groups based on the most commonly diagnosed category (asphyxia); asphyxia and non-asphyxia. Non-asphyctic causes included placentitis, alveolitis, omphalorrhagia, intrauterine iodine deficiency, multiple COD and DNR. The case definitions for each of these individual UCOD and PCOD are shown in Table 1.

2.9. Trace element analysis

The serum trace element analyses were carried out in two external laboratories (Idexx Diavet, Bäch; Labor Zentral, Geuensee; Switzerland). The selenium and total iodine concentrations were measured by inductively coupled plasma-mass spectrometry (MS 820, Varian, Australia; Aurora M90, Bruker, USA) and the copper and zinc analyses were performed photometrically (AU5800; Beckman Coulter, USA).

2.10. Genetic analysis

The inbreeding coefficient (IC) was estimated for pure-bred matings only, as cross-breeds were assumed to have no inbreeding. A total of 33 PP and six MP Holstein cows could be included. The calculation is based on the full pedigree including all registered animals in the herd book, which was provided by the breeding association. The IC for each individual was calculated using the formula $F_I = \sum\left(\frac{1}{2}\right)^n * (1 + F_A)$, with F_A being the

IC of the common ancestor and n the number individuals in the maternal and paternal path including the parents [29].

2.11. Incidence of PM during the study period

Data on the PM rate (0 to 48 hours) in the recruited herds during the period of study and the parity of the dam, calving age, breed of the dam and calf, number of calves, and degree of calving assistance (slight, severe, caesarean section, no answer) were retrieved from the national database (www.agate.ch). In addition, national-level data on PM rate and dam breed were retrieved from the national database for 2016 to 2018.

2.12. Statistical analysis

The continuous variables (investigated cases per farm, herd size, AFC, BCS, gestation length, body weight of calves, maternal blood trace element concentrations, placental weight, crown-rump length, number of cotyledons, minimal/maximal diameter of cotyledons, thyroidal weight, inbreeding coefficient) were described using median and quartiles. The categorical variables (breed, housing type, calving area, calving observation, usage of calving aids, parity, insemination technique, usage of sexed semen, calving induction, udder preparation, calving assistance, time of calving assistance in relation to stage 2, dystocia/history of dystocia, number and sex of calves, defined necropsy findings, placentitis, evidence of pathogen infection, PM rate, time of death) were described using frequencies and proportions. The variables UCOD and PCOD were binary coded, after categorizing each in two main groups (UCOD: infection and non-infection; PCOD: asphyxia and non-asphyxia). Logistic regression models employing backward selection were used for these outcome variables to determine the significant influencing factors: parity (PP vs. MP), gestation length (267 to 273 d, 274 to 278 d, 279 to 284 d and ≥ 285 d), duration of stage two of calving (≤ 1 h, > 1 to 2 h, > 2 to 3 h, > 3 h, no answer possible), calving supervision/observation (yes vs. no), movement of the dam to the calving area prior to calving (never, 0 to 1 d, 2 d, more than 2 d) and dystocia (yes vs. no). Since repeated observations within a farm with more than one PM are possibly clustered, more general mixed models were also applied. If the mixed models did not give a solution, ordinary logistic regression models were

applied. The variable TOD was coded as ordinal. The impact factors on this variable were analysed with nonparametric mixed models. The risk of PM was investigated by logistic regression modelling with backward selection. Pairwise comparisons of groups were Bonferroni-adjusted for multiple comparisons. A p-value of less than 0.05 indicated a significant result. Due to the limited number of twin calves (n=4), these were excluded from statistical analyses. Data were analysed using the statistical software SAS® version 9.4 (SAS Institute Inc., Cary, NC, USA, www.sas.com/).

3. Results

3.1. Farm characteristics

The cases originated from 21 farms in the cantons of Bern (38 cases), Fribourg (7 cases) and Lucerne (2 cases). The median (min, max) number of investigated cases per farm was 1 (1, 12). The median (interquartile range) herd size (no. lactating cows) was 40 (25, 47), main breed was Holstein (85.7%), followed by Braunvieh (9.5%) and Fleckvieh (4.8%) and 57.1% of farms kept their animals in tie-stalls. Half of the farmers used calving boxes and half of them moved pregnant cows to the calving area within 24 hours of calving. Eighty-six percent of the farmers observed cows in the calving area at least every four hours during the day and 18% also every four hours at night. Only one farmer used a calf puller.

3.2. Case data

3.2.1. Dams, calvings and calves

The following data refer to the examined PM calves, their dams and calvings only. The majority (82.2%) of dams were PP, Holstein-Friesian (86.7%) and were sired by Holstein-Friesian bulls (80.0%) by artificial insemination (77.8%). Sexed semen was used in 11.1%. The majority of dams calved without calving induction (93.3%) with good udder preparation (84.4%). The median (interquartile range) AFC and BCS after calving was 26 months (25 months, 28 months) and 3.25 (3, 3.5), respectively. The majority (93.3%) of calvings was observed of which 80.0% were assisted (one person 68.9%; more than one person/veterinarian 11.1%). The majority (68.9%) of cows was given at least two hours after the appearance of the amnion/legs to calve unassisted. Dystocia was recorded in 35.6% of calvings, primarily due to malpositions (81.3%). None of the MP cows had a history of dystocia in the previous calving/s. The median (interquartile range) gestation length and body weight of calves [the majority of which were singletons (91.5%) and female (59.6%)] after death was 281 days (277 days, 284 days) and 38.9 kg (32.9 kg, 43.7 kg), respectively. The farmers judged the majority (83.0%) of calves to be stillborn. Agonal clinical signs were observed in only 6.4% of calves. The median (interquartile range) maternal blood selenium, iodine, zinc and copper concentrations were 56 ug/l (44.0 ug/l, 64.0 ug/l; reference: 51-85 ug/l), 0.8 umol/l (0.6 umol/l, 1.0

umol/l; reference: 0.4-0.9 umol/l), 9.8 umol/l (8.9 umol/l, 11.3 umol/l; reference: 10.7-20.0 umol/l) and 11.6 umol/l (10.4 umol/l, 13.0 umol/l; reference: 7.0-19.9 umol/l), respectively [30-32].

3.2.2. Necropsy and histopathology findings

The median (interquartile range) placental weight, crown-rump length, number of cotyledons, minimal and maximal diameter of cotyledons were 4.3 kg (3.5 kg, 5.7 kg), 101 cm (94 cm, 105 cm), 44 (29, 74), 3 cm (2 cm, 4 cm) and 12 cm (10 cm, 14 cm), respectively. Median (interquartile range) thyroidal weight was 17.0 g (15.1 g, 18.5 g); one calf had a bilaterally diffusely enlarged thyroid gland weighing 33.5 g. This gland showed histological changes compatible with colloid goitre (moderately/markedly enlarged colloid–filled follicles lined mainly by flat columnar epithelium).

The following observations were made during necropsies: Complete pulmonary atelectasis (66.0% of calves), foetal fluid aspiration (45.7%), macroscopically (and/or histologically) visible meconium (15.4%), alveolitis/bronchopneumonia (10.9%), lymphocytic epicarditis (8.7%), subcutaneous limb haemorrhages (5.2%), haemocysts (4.3%), alveolar haemorrhage (2.1%) and intraabdominal bleeding from umbilical arteries (2.1%). Placentitis (mild to marked) was detected in 31.1% of all cases. In one case (qPCR positive for *Neospora caninum*), a multifocal lymphohistiocytic encephalitis was evident histologically.

3.3. Serological and microbiological findings

Pathogen detection and serology results are shown in Table 2 and 3. Indirect evidence of pathogenic infection (Ab-ELISA, MAT) was detected in 66.7% of herds and in 53.3% of dams. Eight cows had exposure to more than one pathogen. Direct evidence of infection (Ag-ELISA, culture, qPCR in combination with histopathology results) was detected in 57.1% of herds, and in 44.7% of calves. All four twin calves were infected. Cows with negative or suspect positive serology results and positive antigen detection (qPCR) were retested 3 to 4 weeks after first sampling. In the case of *Coxiella burnetii* (n = 7) the titre (determined as antibody level expressed as S/P value) increased in two cows (one each from negative to positive and from suspect positive to positive) and

decreased in one cow (from suspect positive to negative). The titre remained negative in four cows. In the case of *Leptospira* spp., the titre did not change in any of the three cows retested.

Co-infections were detected in four calves; one calf was positive for *Neospora caninum* (qPCR) and *Coxiella burnetii* (qPCR); in two calves (twins) *Streptococcus pluranimalium* (culture) was found together with *Coxiella burnetii* (qPCR) and in one calf, pathogenic *Leptospira* spp. (qPCR) and *Coxiella burnetii* (qPCR) were detected.

3.3.1. High-risk herd-level data

The following data were retrieved from the Swiss animal movement database and refer to all calves born on the farms included in the study within the sampling period. There were 1,003 calvings (45.1% PP cows) with a total of 1,029 calves. At the farm-level, the median (min, max) PM rate was 10.0% (5.3%, 28.2%). Twin calving was rare in PP (0.9%) and MP (3.8%) cows. At the cow-level, the PM rate (single calves, twins) was 11.5% (10.6%, 28.0%). In PP and MP cows, the PM rate was 19.7% (18.8%, 75.0%) and 4.9% (3.8%, 19.0%), respectively. The multiple logistic regression showed that PM rate was not affected by farm (P = 0.7587), calving age (P = 0.9994), breed of the dam (P = 0.9507) nor breed of the calf (P = 0.0898). However, PP cows were more likely to have PM than MP cows (P < 0.0001). The median (interquartile range) AFC was 27 months (25 months, 29 months) and the majority (87.3%) of PP cows had an AFC ≥ 24 months. The PM rate was higher when cows had an AFC < 24 months (OR = 3.159; 95% CI: 1.505-6.633) compared to those with an AFC ≥ 24 months. The modelled probability for PM was 36.8% for an AFC < 24 months and 16.1% for an AFC ≥ 24 months. The odds ratio for PM was higher in cows with an AFC < 24 months (OR = 16.003; 95% CI: 6.745-37.968) than in cows with an AFC ≥ 24 (OR = 5.066; 95% CI: 2.671-9.607). Severe calving assistance occurred in 6.8% of all PP and in 3.6% of all MP cows and severe assistance occurred more often in male calves (overall: 5.8%; PP cows: 3.5%; MP cows: 2.3%) than in female calves (overall: 4.6%; PP cows: 3.3%; MP cows: 1.3%). More than half (52.9%) of the calves were female (PP cows: 56.1%; MP cows: 50.3%). The PM rate was higher for male calves than for female calves (OR = 1.61; 95% CI: 1.050-2.475), (P = 0.0291). Only 18.1% of calves were cross-bred. There

was a lower PM rate (6.3%) in cross-bred calves compared with pure-breds such as Holsteins (13.5%).

3.3.2. Swiss national data

The overall PM rate, across all breeds, within 48 hours of birth in 2016, 2017, and 2018 was 2.2%, 2.2% and 2.3%, respectively. Over 90% of those calves died within the first 24 hours of life. Unfortunately, differentiation between PP and MP was not possible and as a consequence, the AFC could not be evaluated either. Red Holsteins (8.8%, 7.0%, 6.1%) had higher PM rates than Holsteins (3.0%, 2.9%, 3.1%). The Brown Swiss breed had PM rates of 1.7%, 1.7% and 1.8%, whereas the Original Braunvieh had PM rates of 6.1%, 4.0% and 4.1%. Fleckvieh had a PM rate of 1.6% in all three years and cross-breeds had a PM rate of 1.9%, 1.7% and 2.1%.

3.4. Time of death

Based on the pathological examination, TOD was classified as intrapartum in the majority of cases (68.1%), followed by prepartum (21.3%) and postpartum (10.6%). Univariate analyses in the nonparametric mixed models revealed that parity, calving supervision, dystocia and degree of autolysis significantly influenced this variable (Table 4). There was an interaction between parity group and TOD; PM in PP cows occurred more often prepartum (n = 7 vs. 1) and intrapartum (n = 29 vs. 3), whereas in MP cows the calves died more often postpartum (n = 4 vs. 1), (P = 0.0051). Calves from supervised calvings died more often prepartum (n = 8 vs. 0) and intrapartum (n = 31 vs. 1) than calves from non-supervised calvings which died mostly postpartum (n = 2 vs. 3), (P = 0.0258). Calves which died prepartum were more likely to be born in dystocia (n = 5 vs. 3), (P = 0.0320). Calves with moderate or severe autolysis were more likely to have died prepartum than calves with slight or no autolysis (7 vs. 1 vs. 0), (P = 0.0165). Significant variables influencing TOD could not be detected in the final mixed or logistic regression models.

3.5. Cause of death

The UCOD diagnoses are shown in Table 5. The most common UCOD was infection. No significant associations could be detected between the explanatory variables tested (Table 4) and the UCOD categories in mixed models (no solutions) or in the logistic regression models. Summarized values per farm (only one explanatory variable) using nonparametric methods did not show any significant relationships either. The PCOD diagnoses are shown in Table 5. The most common PCOD was asphyxia. No significant associations could be detected between the explanatory variables tested (Table 4) and the PCOD categories in mixed models (no solutions) or in the logistic regression models. Summarized values per farm (only one explanatory variable) using nonparametric methods did not show any significant relationships either.

3.6. Genetic analysis

The IC was estimated for all purebred Holstein calves (33 calves from PP and six from MP cows). The overall median (interquartile range) IC was 0.059 (0.046, 0.069), with 0.059 (0.045, 0.075) for PP and 0.056 (0.050, 0.064) for MP calves. The IC ranged from zero, where the dam or the sire was unknown, up to 0.114.

4. Discussion

The investigative model employed here yielded high quality epidemiological and pathological data on the factors associated with, and the timing and causes of, bovine perinatal mortality. Despite perceptions by Swiss farmers and veterinarians, the PM rate on Swiss farms nationally is low and does not appear to have changed in the last decade as evidenced by the PM rate reported here for 2016 to 2018 (~2.3%), and that reported by Bleul [6] in 2011 (2.4%). The low PM rate reflects the small herd sizes, the high value of calves (and associated good calving management) and the rare use of mechanical calving aids (calf pullers), thus avoiding iatrogenic fatal parturient trauma.

However, the PM rate on the high-risk farms in this study was five-times higher (11.5%). All of the enrolled farms met the inclusion criterion (threshold of PM > 5%) throughout the study period indicating that high-risk farms turned out to be high PM rate farms. This indicates a high level of record keeping and a low level of 'farm blindness' [Mee, in press]. The high PM rate on these farms may be explained by their self-selection as high risk farms, the associated significant risk factors on these farms and the results from the pathological investigations.

While statistically significant risk factors associated with PM were detected, some of them did not contribute greatly to explaining the overall high PM rate due to their low occurrence in the population (e.g. twins or primiparae with an AFC < 24 months), while others were more relevant. For example, there were both an abnormally high proportion of calves born to primiparae (45%) and of calves which died in the perinatal period, and were examined in this study, born to primiparae (80%). This parity bias would contribute to a high PM rate given that parity is a significant risk factor for PM [7].

Though AFC less than 24 months is associated with higher PM rates [33], the median AFC in this study was 27 months suggesting that though a statistically significant risk factor, it was not a major contributor to the high herd-level PM rate. This was confirmed where the modelled probability for PM was 36.8% for an AFC < 24 months compared to 16.1% for an AFC ≥ 24 months. However, only 9.7% (3/31) of heifers with an AFC < 24 months needed severe calving assistance and this was mainly due to malposition, not foeto-pelvic disproportion, suggesting that AFC *per se* was not an important risk factor at the herd-level.

Even though the PM rate was higher for male than female calves in these herds, the majority of calves born were female (53%) suggesting that at the herd-level gender did not contribute significantly to the high overall PM rate. However, more female than male PM calves were submitted for examination. This may be partly explained by greater female:male ratio. This distribution is in contrast with that of Bleul [6] in a previous Swiss PM study. It is suggested that these two female biases may be explained by increasing usage of sexed semen within the last decade and the (associated) higher value of female calves.

The majority (82%) of calves born in the study herds were purebred Holsteins (Holstein and Red Holstein) and these calves had a high PM rate (13.5%). This is 3-fold higher than the Swiss average in the last three years and also higher than that described by Bleul [6]. This high PM rate in Holstein calves may reflect the high PM rates on these high risk farms, but our results might also support an association between the introgression of North American Holstein sires' genes and high stillbirth rate [7,34,35].

Excessive inbreeding has been associated with dystocia and/or stillbirth, especially in primiparae [33,36,37]. The median IC in this study (0.059; 0-0.114) is similar to values reported previously, though the maximum value is quite high. The IC for Holsteins internationally varies between 0.04 and 0.06 (0.057 and 0.042 in pedigree Swiss Holstein-Friesians and Red Holsteins, respectively), [38]. The lower PM rate in cross-bred calves than in pure-bred calves may reflect an aspect of hybrid vigour in cross-breds.

In this study housing type (tie and free stall) was not a significant risk factor for PM and the results from studies to date are conflicting. Gulliksen et al. [39] observed lower stillbirth rates in tie-stalls, attributed to better calving supervision. In contrast, higher stillbirth rates in tie-stalls were associated with higher rates of dystocia due to inadequate mobility and psychological stress in tie-stalls compared to free stalls [40].

The larger than Swiss average herd sizes on both tie- and free stall farms in this study is unlikely to have contributed to higher rates of PM. In 2018, the average dairy herd size (cows only) in Switzerland was 20 cows [41], while in this study the average herd size was twice as large (40 cows). While large herd size has been reported as a risk factor for PM in some [6,39], but not all studies [42] attributed to insufficient monitoring

around parturition and a high number of unassisted calvings [39], in this study 93% of calvings were observed. This suggests that PM rate may be scale-neutral but management-dependent.

Calving management (calving movement, calving location, calving supervision, calving intervention policies and calving assistance) can have a significant effect on PM [12].

The timing of movement of cows from the dry to the maternity accommodation can affect calving duration and PM rate [12]. Only two farmers moved cows within 1 to 2 days prior to calving into the box as recommended by Mee [43]. Most of the other farmers moved their animals less than one day prior to calving, which carries the risk of moving the animals during stage 1 of calving leading to higher dystocia and PM rates [12].

The quality and frequency of calving supervision can influence PM rate [12]. The majority of farmers monitored calvings every 2 to 4 hours during daytime, but at more than 4 hour intervals at night-time. Monitoring cows every 3 to 6 hours after the onset of stage 1 of calving is recommended [43]. Prolonged intervals between observations may lead to undetected bradytocia with fatal sequelae. However, necropsy examinations did not detect bradytocia as a major cause of PM in these herds.

The majority of farmers followed current recommendations on timing of calving assistance (80-120 minutes after onset of stage 2) [10,44]. This, along with limited use of mechanical calving aids, may be the reason for the extremely low contribution of dystocia to the causes of death in these PM calves.

In this study we used two independent assessments of TOD; by the farmer and by the pathologist. Interestingly, this produced widely divergent results. Farmers recorded a stillbirth in 83% of cases, whereas the necropsy confirmed this in only 20% of cases (on the basis of pulmonary ventilation). This discordance is surprising given that farmers observed 93% of PM calvings, but perhaps reflects a more lax definition of stillbirth amongst farmers (e.g. died/dead ‘at’ birth, may include before, during birth and immediately after birth) and the fact that calves dying intrapartum may have small areas of lung inflation. If farmers overestimate/misdiagnose ‘stillbirth’ they may be less likely to attempt to assist calvings of at-risk foetuses promptly or revive apparently stillborn calves that in fact have (partially) inflated lungs and may respond to resuscitation.

Furthermore, parity, supervision of the calving and dystocia were factors significantly influencing TOD.

While risk factors demonstrate significant associations with PM, they do not explain the causes of the high PM rates on these farms. This challenge was addressed by answering both *why* and *how* calves died to determine the UCOD and the PCOD, respectively. From these analyses it is clear that the most important UCOD contributing to the high PM rate on these farms was foetal infection. In total, 44.7% of all PM calves had direct evidence of pathogenic infection. This is more than four-times higher than that reported recently in study of the causes of PM on Polish dairy farms [45]. The reason for this high infection rate may lie in the high prevalence of some of the detected pathogens in Swiss dairy herds (e.g. *Coxiella burnetii)* but a contributory reason is the submission bias in the study. Farmers were more likely to submit calves whose death they could not explain. Thus, because infected calves do not exhibit pathognomonic clinical signs (only 6% of calves showed any clinical signs) they were more likely to be submitted, hence the high proportion of infected calves contributing to the UCOD and high PM rate.

Coxiella burnetii was the most frequently detected pathogen (32% of calves, by qPCR), but only 15% of placentae and 19% of calves had histological signs of inflammation (placentitis and bronchopneumonia), respectively. These findings suggest that acute infections with *Coxiella burnetii,* or other pathogens, may occur without histological lesions. Additionally, mild, histologically detectable placentitis may be physiologically normal during pregnancy and after delivery [46]. The detection of *Coxiella burnetii* by qPCR on 38% of farms is in agreement with the increasing occurrence of *Coxiella burnetii* in livestock throughout Switzerland [47]. While a similar maternal *Coxiella burnetii* seroprevalance, as found here (13%) has been reported previously for bovine abortions in Switzerland (15.9%); the detection of *Coxiella burnetii* by qPCR in placentae (11.6%) and AC (12.3%) was much lower [22].

Coxiella burnetii was detected in the four calves (two of which were twins) with co-infections. Co-infection with this pathogen was observed in 13.7% of abortions in a recent Brazilian study [48]. We suggest that *Coxiella burnetii* is not only important as a primary infectious agent, but also as a possible factor enhancing co-infections with other

pathogens. An additional explanation could be, that co-infections are more likely to occur in countries where classical abortive agents (e.g. *Brucella*) do not play a role, as in Switzerland [49].

The role of *Parachlamydia* spp. and *Chlamydia*-like organisms in the aetiology of bovine PM is unclear; two studies described a possible association with bovine abortions [50,51], but not PM. Of the five cases of PM where these organisms were detected here, three cases had inflammatory lesions (placentitis, alveolitis), while two cases were histologically normal.

These findings on pathogens highlight the difficulties in assigning a COD to an infectious cause. It is recommended that evaluation of true infectious causes should focus on both antigen detection and histological examination.

In less than 10% of cases, a non-infectious UCOD was diagnosed, e.g. dystocia. This is a surprising finding as dystocia has traditionally been diagnosed as a major cause of PM [3,1,6,13,14]. However, as discussed previously, the submission bias in this study, the infrequent use of mechanical calving aids, the excellent calving supervision and high value of calves all probably significantly reduced the representation of dystocia cases in the final categorisation.

Despite detailed case histories and extensive necropsy and laboratory examinations, in over half of all cases a UCOD could not be assigned (diagnosis not reached = DNR). In examining the PCOD it is clear that in many of these cases the foetus suffered asphyxia before or during calving but the ultimate cause of this asphyxia could not be determined, hence the high DNR - UCOD. This is the essential difference between the ultimate and proximate COD; while one may be able to determine that the foetus died because of asphyxia in many cases the ultimate cause of this proximate condition could not be determined (asphyxia of unknown origin). The high UCOD - DNR rate found here is primarily due to the absence of typically more common causes of PM in this unusual calf submission cohort (i.e. dystocia) and the categorisation of asphyxia as a PCOD, not a UCOD. In many necropsy studies on PM both asphyxia and dystocia are categorised together [50,51].

Asphyxia was the most frequently diagnosed (44.7%) PCOD; 66% of calves had complete pulmonary atelectasis. This is in agreement with other studies on PM in calves

[2,9]. While it has traditionally been closely associated with dystocia (often used as a synonym for traumotocia) it also occurs in bradytocia, eutocia and 'non-clinical dystocia' (clinically undetectable prolonged or abnormal stage one or two of calving) [7]. Multiple COD was the next most commonly diagnosed PCOD (21.3%). This is not surprising given the investigative SOP and unusually detailed research-level investigations conducted in each of these cases of PM. This level of investigation would not be typical of that conducted during a veterinary practitioner on-farm necropsy or routine necropsy in a diagnostic veterinary laboratory. In some of these cases it is possible that infectious and non-infectious diagnoses were made, and that these were interrelated, e.g. infection with pathogenic *Leptospira* spp. and foetal fluid aspiration. However, these could not be disaggregated as both of these criteria for diagnosis can also occur independently; they are neither mutually inclusive nor mutually exclusive. Placentitis was the third most commonly diagnosed PCOD (12.8%). This might be expected given the high prevalence of exposure to infection in these herds (67%), dams (53%) and calves (45%). The DNR rate (14.9%) for PCOD was much lower than that for UCOD (57.5%). This reflects the dichotomy between these diagnostic frameworks in the way COD are attributed. In the former, terminal conditions or lesions causing death need to be detected while in the latter, the ultimate cause of these needs to be defined; the former can be accomplished more often especially, as found here, when a research-level investigation is conducted. Despite the detailed investigations of each case, significant risk factors associated with either the UCOD or the PCOD could not be determined. This was probably due to the small dataset (and high proportion of multiple COD) and the consequent decision to aggregate the COD into binary groups; infectious/non-infectious and asphyxia/non-asphyxia.

Prepartum maternal dietary trace element imbalances (selenium, zinc, copper, iodine) are associated with PM and poor calf performance [12,57,58]. However, due to case selection after calving, sampling of case dams' prepartum was not feasible here; dams were sampled 0 to 23 days postpartum. Differences between mineral supplementation in lactating and dry cows/heifers can lead to an increase in trace element status postpartum, thus samples collected after calving may overestimate precalving maternal (and foetal) trace element status. Despite this, the selenium (especially MP cows) and

zinc (PP and MP cows) status of the dams of PM calves [whether collected 0 to 1 (n=13) or 0 to 23 days postpartum] tended to be marginal or deficient, whereas iodine and copper values were generally normal (reference values according to Mehdi et al., Kraft and Dürr, and Spolders et al. [30-32]).

The selenium findings may be explained by the low selenium status of Swiss soils [56], a factor reflected in the low selenium status of newborn calves in Switzerland [57]. In the present study, only approximately half of all farmers supplemented their pregnant animals' diet with adequate selenium (≥ 40mg/kg DM). This probably reflects a lack of farmers' awareness of the importance of selenium [57]. Nevertheless, it cannot be inferred *per se* that there is a deficiency in the calf if the cow is undersupplied, as selenium is partitioned to the foetus during mid and late gestation [58]. This may be one of the reasons why, despite low maternal blood selenium values, none of the PM calves showed gross or histological signs of muscular dystrophy. Additionally, if the vitamin E status of pregnant cows is adequate and selenium undersupply is not severe, the calf may not be affected.

While epidemiological studies have associated low zinc status with young calf morbidity [54], there is no evidence of zinc deficiency causing PM. Hence, the low blood zinc concentrations found here may be incidental.

One PM calf had colloid goitre, with uninflated lungs and some alveolar haemorrhage. Calves with an abnormal thyroid gland are significantly more likely to have uninflated lungs, [55]. However, the postpartum serum iodine concentration in the dam was normal (1.12 umol/l), as in 42 of 45 tested animals. This is perhaps not surprising given that the dam was sampled 13 days after calving (she also had a normal blood selenium status: 55 ug/l). Colloid goitre represents the involutionary phase of hyperplastic goitre after iodine status has been restored.

There were inherent biases and limitations in this study. The selection of cases was biased by the farmers, as mostly more valuable female calves and unexplainable deaths were referred for further examinations. This probably led to the non-representative distribution of the COD. The limited number of cases examined precludes high confidence in the strength of associations between risk factors and PM and estimates of the relative prevalence of causes of death. The majority of the cases

were from the canton of Bern probably due to the geographical proximity to the Institute of Animal Pathology in Bern. However, this canton has the highest number of cattle in Switzerland (approx. 20% of the 1.5 million head of cattle) [59] and so the results are relevant to this population.

Despite the study limitations, the investigative model and research methods used in this study have external validity which can be replicated and re-evaluated, respectively, in future studies on PM internationally. Specifically, the strengths of the model are the definition of a case, collection of epidemiological data on the farm management relevant to calf mortality, the sampling of the dams of affected calves, the detailed necropsy and laboratory examinations and the synthesis of these diverse strands of investigation into a detailed picture of *when, why* and *how* calves die in the perinatal period. This is a multi-disciplinary process involving farmers, veterinary practitioners and veterinary pathologists.

For future investigation of problem herds additional options which could be considered include random sampling, sampling of healthy controls and whole herd sampling. While a 'random' sample of cases would be less expensive to investigate, given the low incidence of perinatal mortality it may be impractical in small herds (e.g. with a 5% loss rate in a 50-cow herd there is only two to three cases from which to sample) but may have application in larger herds (e.g. 5% loss; 500-cow; 25 cases). Cohort (controls) sampling has merit for maternal sero-surveillance of infectious diseases and mineral disorders. Ideally, it is recommended that a whole herd, prospective, active surveillance model be overlaid on this investigative model. As outlined above, in passive surveillance models the farmer decides which calves to submit. This submission bias can significantly affect the investigative and surveillance outcomes even where a detailed investigative model, such as evaluated here, is employed. For example, fewer, more visible (as opposed to occult) and lethal congenital defect cases are submitted in passive compared to active surveillance models [7]. However, the greater investigative yield of whole herd sampling needs to be balanced against the additional costs which may accrue.

5. Conclusions

It is concluded from this research study that while the PM rate in Swiss dairy herds is currently low by international standards, high PM rate herds do occur. In this study of 21 such herds some of the risk factors associated with high PM rates, such as parity, were expected while others, for example infection, were not, being more associated with abortion, not PM. Specifically, there was a high herd- and animal-level prevalence of *Coxiella burnetii* and associated lesions in the dead calves and their placentae indicating that this was an important contributory cause of the high PM rates in these herds. Surprisingly, dystocia was not a major cause of PM, a reflection of both calving management and submission bias, but asphyxia was a common finding in these calves, the majority of which died intrapartum. It was also concluded that the investigative model and research methods used in this study could act as exemplars for future studies on PM internationally.

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Conflict of interest

All authors rule out any conflict of interest regarding this work.

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Highlights

- The perinatal mortality (PM) rate in this study was 10% at the farm-level, and at the cow-level, 11.5%. These values, from high-risk farms, were approximately five-times higher than the contemporary national bovine PM rate (2.3%) in Switzerland.
- The majority of calves died intrapartum. The most commonly diagnosed initiating/ultimate cause of death (UCOD) was infection of which *Coxiella burnetii* was the most frequently detected pathogen, by antigen.
- The investigative model and research methods used in this study have external validity, which can be replicated and re-evaluated, respectively, in future studies on PM internationally.

Table 1: Criteria used to assign causes of death (COD)

Cause of death	Criteria
Asphyxia	Gross: complete or partial atelectasis; lungs appear dark purple, moist and heavy.
	Histology: moderate/marked amniotic fluid aspiration (± meconium aspiration); moderate/marked, multifocal keratinocytes ± exfoliated epithelia, ± yellow/brown granular material (meconium) and eosinophilic fine granular material in alveoli and bronchioles, ± congested, leukocytostatic capillaries.
Alveolitis	Histology: moderate/marked, diffuse increase in intra-alveolar neutrophils, macrophages with accompanying leukocytostasis.
Colloid goitre	Gross: absolute increased thyroid gland weight (>30g).
	Histology: moderately/markedly enlarged colloid–filled follicles lined mainly by flat columnar epithelium.
BVD	Detection of BVDV by antigen ELISA in ear-notch sample.
IBR	Detection of BHV-1 by antibody ELISA in dam serum.
Brucellosis	Detection of *B.abortus* positive culture and moderate/marked, multifocal, purulent, necrotising placentitis.
Coxiellosis	Detection of *C.burnetii* DNA by qPCR in placenta and/or abomasum contents and moderate/marked, multifocal, purulent, necrotising placentitis. Or, detection of *C.burnetii* DNA without placentitis indicating very acute, recent infection.
Chlamydiosis	Detection of *C.abortus* DNA by qPCR and sequencing in placenta and/or abomasum contents and moderate/marked, multifocal, purulent, necrotising placentitis. Or, detection of *C.abortus* DNA without placentitis indicating very acute, recent infection.
Leptospirosis	Detection of pathogenic *Leptospira* spp. DNA by qPCR in placenta and/or abomasum contents, and moderate/marked, multifocal, purulent, necrotising placentitis. Or, detection of pathogenic *Leptospira* spp. DNA without placentitis indicating very acute, recent infection.
Neosporosis	Detection of *N.caninum* DNA by qPCR in the foetal brain and focal or multifocal encephalitis
Broad-spectrum bacterial and fungal pathogens[a]	Large numbers and/or pure culture in the foetal abomasum and/or other tissues, associated placentitis and exclusion of other common abortigenic agents.
Dystocia	Anamnesis: history of severe calving assistance (e.g. relative foetal oversize and/or foetal maldisposition) and/or bradytocia (prolonged calving),
	Gross: multifocal, subcutaneous limb petechial haemorrhages, moderate/marked acute, diffuse pulmonary congestion.
	Histology: multifocal alveolar haemorrhages and capillary congestion.
Multiple COD	Combinations of the findings from the multiple causes of death listed in this table found in the same foetus/calf
Omphalorrhagia	Marked, acute haemoabdomen (up to 2L of free blood and coagulum) with one or both intra-abdominal umbilical arteries patent and accompanying carcass anaemia (e.g. pallor of mucous membranes)
Placentitis	Histology: moderate-high grade, acute, focal-diffuse, cotyledonary +/- intercotyledonary, villar/interstitial, necrosis, inflammation (neutrophil, macrophage, lymphocyte infiltration), edema, +/- vasculitis, +/- dystrophic calcification/mineralisation, +/- autolysis
Premature placental separation	Anamnesis: placenta expelled with/immediately after the foetus. Gross: the placenta may be normal.
	Histology: acute, multifocal haemorrhage into the interstitium of the cotyledonary villi.

[a] Among others: *Mucor species, Streptococcus pluranimalium, Streptococcus uberis, Clorstridium perfringens, Escherichia coli, Campylobacter foetus* subsp. *foetus/venerealis, Salmonella* spp.

Table 2: Pathogen antigen (bacterial, parasitic and viral) detection in 47 cases of perinatal mortality from 21 Swiss dairy herds

Pathogen	Detection method	Calves from PP cows	Calves from MP cows	Total		Total detection %
		No. (%)	No. (%)		Farm no.	
BVDV	Ag-ELISA	0/38[a]	0/9	0/47	-	0
Neospora caninum	Real-time PCR [c]	1/38 (2.6%)	0/9	1/47	10[1]	2.1
Brucella abortus	Culture	0/38	0/9	0/47	-	0
Coxiella burnetii	Real-time PCR [d]	10/38 (34.2%)	5/9 (55.6%)	15/47	7, 10[1], 3[b], 7, 13, 14,15, 7[2,b], 18, 7, 3[3], 22	31.9
Chlamydia abortus	Real-time PCR [d, e]	4/38 (10.5%)	1/9 (11.1%)	5/47	3, 5, 6, 12, 6	10.6
Mucor ssp	Culture	3/38 (7.9%)	1/8 (12.5%)	4/47	11, 7, 23, 15	8.5
Streptococcus pluranimalium	Culture	3/38 (7.9%)	0/9	3/47	3, 7[2,b]	6.4
Streptococcus uberis	Culture	2/38 (5.3%)	0/9	2/47	3, 3	4.3
Clostridium perfringens	Culture	1/38 (2.6%)	0/9	1/47	22	2.1
Escherichia coli	Culture	1/38 (2.6%)	1/9 (11.1%)	2/47	7, 7	4.3
Campylobacter foetus subsp. *foetus*	Culture	0/38	0/9	0/47	-	0
Campylobacter foetus subsp. *venerealis*	Culture	0/38	0/9	0/47	-	0
Salmonella sp.	Culture	0/38	0/9	0/47	-	0
Pathogenic *Leptospira* spp.	Real-time PCR [d]	2/38 (5.2%)	1/8 (12.5%)	3/45	21, 7[3], 6	6.7

[1-3)] Four calves with coinfection; 1) *Coxiella burnetii* and *Neospora caninum*; 2) *Coxiella burnetii* and *Streptococcus pluranimalium*; 3) *Coxiella burnetii* and pathogenic *Leptospira* spp.

[a] Number of calves positive to at least one pathogen / total number of calves tested

[b] Twins; Both calves tested positive

[c] This calf also had histological multifocal lymphohistiocytic encephalitis

[d] Antigen detection in placenta, foetal abomasum contents or both

[e] The sequencing of the positive results revealed *Chlamydiales* (n = 4; all in primiparous cows) and *Parachlamydia* (n = 1; multiparous cow)

BVDV = Bovine viral diarrhoea virus; Ag = antigen

Table 3: Pathogen antibody (viral and bacterial) detection in the dams of 47 cases of perinatal mortality from 21 Swiss dairy herds

Pathogen	Detection method	No[a]	PP cows	No[a]	MP cows	No[a]		Total detection (%)
			Positive					
			Cows % (titre)				Farm no.	
BHV-1	Ab-ELISA	0/37	0	0/8	0	0/45		0
Brucella abortus	Ab-ELISA	0/37	0	0/8	0	0/45		0
Coxiella burnetii	Ab-ELISA	3/37	8.1 (n = 1; S/P >40%) (n = 2; S/P >30%; <40%)	3/8	37.5 (n = 3; S/P >40%)	6/45	3, 7[1], 3[4], 3[4], 3, 18 (n = 4; S/P >40%) (n = 2; S/P >30%; <40%)	13.3
Chlamydia abortus	Ab-ELISA	14/37	37.8 (n = 10; S/P >40%) (n = 4; S/P >30%; <40%)	3/8	37.5 (n = 3; S/P >40%)	17/45	11[5], 3[4], 3[4], 7[1], 15, 7[2], 12, 6, 6, 6, 3, 7, 2, 3, 8, 7, 19 (n = 10; S/P >40%) (n = 4; S/P >30%; <40%)	37.8
L. Grippothyphosa	MAT	1/37	2.7	0/8	0	1/45	7[2]	2.2
L. Australis	MAT	1/37	2.7	0/8	0	1/45	13[8]	2.2
L. Pomona	MAT	0/37	0	0/8	0	0/45	-	0
L. Tarassovi	MAT	0/37	0	0/8	0	0/45	-	0
L. Canicola	MAT	0/37	0	0/8	0	0/45	-	0
L. Icterohaemorrhagica	MAT	0/37	0	0/8	0	0/45	-	0
L. Hardjo	MAT	3/37	8.1 (n = 3; 1:400)	0/8	0	3/45	11[5], 9[3], 5[6] (n = 3; 1:400)	6.7
L. Sejroe	MAT	5/37	13.5 (n = 3; 1:100) (n = 2; 1:200)	0/8	0	5/47	9[3], 5[6], 13[8], 11[5], 10[7] (n = 3; 1:100) (n = 2; 1:200)	10.6
L. Ballum	MAT	1/37	2.7 (1:200)	0/8	0	1/45	10[7] (1:200)	2.2

[1-8)] Eight cows with more than one non-negative titre; the same superscript in different rows indicates the different positive results present in the same cow.

[a] Number of positive cows to the number of all cows tested; BHV-1 = Bovine herpesvirus 1; MAT = microscopic agglutination test; Ab = antibody

Table 4: P-values of the nonparametric mixed models

	TOD	UCOD	PCOD
Variable	P-Value		
Parity	0.0051	0.8342	0.2407
Gestation length	0.1663	0.4000	0.7907
Calving duration	0.9712	0.9461	0.5181
Calving supervision	0.0258	0.4759	0.5426
Dislocation prior to calving	0.3571	0.8067	0.8928
Dystocia	0.0320	0.5263	0.1906
Death to farmer's impression	0.2174	n/a	n/a
Degree of autolysis	0.0165	n/a	n/a

Table 5: Categorization and frequency of observations of ultimate (UCOD) and proximate (PCOD) cause of death in 47 cases of bovine perinatal mortality

Category	Description	%
UCOD	Infection	34.0
	Dystocia	4.3
	PPS[a]	2.1
	Multiple[b]	2.1
	Diagnosis not reached	57.5
PCOD	Asphyxia	44.7
	Placentitis	12.8
	Alveolitis	2.1
	Omphalorrhagia	2.1
	Colloid goitre	2.1
	Multiple[c]	21.3
	Diagnosis not reached	14.9

[a] Premature placental separation

[b] One case with multiple diagnoses for UCOD: prolonged/difficult calving and infection with pathogenic *Leptospira* spp. (qPCR)

[c] Ten cases with multiple diagnoses for PCOD: Infection with pathogenic *Leptospira* spp. (qPCR), and foetal fluid aspiration (n = 1); infection with *Coxiella burnetii* (qPCR), foetal fluid aspiration and histopathological signs of placentitis (n = 4; two of these cases were twins); infection with *Neospora caninum* (qPCR positive and histological signs of meningoencephalitis but no histopathological evidence of placentitis) and *Coxiella burnetii* (qPCR) and foetal fluid aspiration (n = 1); infection with *Coxiella burnetii* (qPCR) and *Streptococcus pluranimalium* (culture), foetal fluid aspiration and histopathological signs of placentitis and epicarditis (n = 1); infection with *Coxiella burnetii* (qPCR) and *Streptococcus uberis* (culture), histopathological signs of placentitis and epicarditis (n = 1); foetal fluid aspiration and histopathologically visible placentitis without any detectable pathogen and zinc deficiency of the dam (n = 2; one of these calves also had epicarditis.