Preventive Veterinary Medicine 181 (2020) 105056

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Associations of barn air quality parameters with ultrasonographic lung lesions, airway inflammation and infection in group-housed calves

Katharina van Leenen[a,*], Jonathan Jouret[b], Peter Demeyer[c], Laura Van Driessche[a], Lieze De Cremer[a], Christien Masmeijer[a], Filip Boyen[d], Piet Deprez[a], Bart Pardon[a]

[a] Department of Large Animal Internal Medicine, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820, Merelbeke, Belgium
[b] Belgian Center for Occupational Hygiene, Tramstraat 59, 9052, Zwijnaarde, Belgium
[c] Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Burgemeester van Gansberghelaan 115 Bus1, 9820, Merelbeke, Belgium
[d] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820, Merelbeke, Belgium

ARTICLE INFO

Keywords:
Temperature
Ammonia
Air velocity
Thoracic ultrasonography
Broncho-Alveolar Lavage fluid
Epithelial cell percentage

ABSTRACT

Barn climate is believed to play a major role in the bovine respiratory disease complex. However, the exact air quality parameters associated with (sub)clinical pneumonia or airway inflammation in calves are currently unknown. The objective of this cross-sectional study was to assess associations of air quality parameters with clinical signs, lung consolidation, pulmonary inflammation and infection in group-housed calves. In total, 60 beef and dairy farms were visited from January to April 2017 and 428 calves sampled. Measured air quality parameters included continuous 24-h measurements of ammonia concentration, relative humidity and temperature and punctual measurements of air velocity, ammonia, CO_2 and bacterial air load. Calf sampling consisted of clinical examination, thoracic ultrasonography and broncho-alveolar lavage sampling for bacteriological and cytological analysis of broncho-alveolar lavage fluid (BALf). Average air temperature was 14.2 °C (standard deviation (SD) 4.4, range 5.5–23.9) and relative humidity 68.8 % (SD 8.9, range 52.2–91.6). Average ammonia concentration was 1.7 ppm (SD 0.9, range 0–10.0). Lung consolidations of ≥1 cm, ≥3 cm and ≥6 cm in depth were present in 41.1 % (176/428), 27.1 % (116/428) and 16.1 % (69/428) of the calves, respectively. Average pen temperature was positively associated with consolidations of ≥1 cm ($P = 0.005$), ≥3 cm ($P = 0.002$) and ≥6 cm ($P < 0.01$). Ammonia exposure, in hours > 4 ppm, was associated with lung consolidation ≥1 cm (odds ratio (OR) = 1.73; confidence interval (CI) = 1.02–3.07; $P = 0.04$). Ammonia concentration was positively associated with BALf epithelial cell percentage ($P = 0.01$). Air velocity > 0.8 m/s was associated with increased odds of lung consolidation of ≥3 cm (OR = 6.8; CI = 1.2–38.5; $P = 0.04$) and ≥6 cm (OR = 15.9; CI = 1.2–200.0; $P = 0.03$). The prevalence of lung consolidations ≥1 cm was higher in the draught (81.8 %; $P = 0.0092$) and warm, dry and ammonia accumulation clusters (54.2 %; $P = 0.02$) compared to the presumably normal cluster (31.6 %). In addition, in the warm, dry and ammonia cluster the prevalence of lung consolidations ≥3 cm (38.1 %; $P = 0.04$) and ≥6 cm (31.4 %; $P = 0.01$) in depth were higher compared to the presumably normal climate cluster (18.2 % and 9.1 %, respectively). Of all frequently measured indoor air quality parameters, only average temperature, ammonia concentration and air velocity were associated with pneumonia and might therefore be preferable for cost-effective evaluation of calf barn climate.

1. Introduction

Bovine respiratory disease (BRD) has major effects on economic results, longevity and antimicrobial consumption in beef, dairy and veal calves (Griffin, 1997; Bach, 2011; Pardon et al., 2013). The disease results from the complex interaction of pathogens, host immunity and environmental risk factors (Ellis, 2001; Griffin et al., 2010; Dubrovsky et al., 2019). Continuing to control the impact of BRD solely by prophylactic and metaphylactic antimicrobial treatment is both incompletely effective and unacceptable in the era of antimicrobial

Abbreviations: BALf, broncho-alveolar lavage fluid; SD, standard deviation; R, range; OR, odds ratio; CI, confidence interval; nBAL, non-endoscopic broncho-alveolar lavage; TNCC, total nucleated cell count; PPLO, pleuro pneumonia-like organism; ROC, receiver operating characteristics; WI-score, Wisconsin score; BRD, bovine respiratory disease

* Corresponding author.
E-mail address: Katharina.vanLeenen@ugent.be (K. van Leenen).

https://doi.org/10.1016/j.prevetmed.2020.105056
Received 9 December 2019; Received in revised form 5 May 2020; Accepted 7 June 2020

resistance in humans and animals, demanding a decrease in antimicrobial consumption (Baptiste and Kyvsgaard, 2017; EMA/EFSA, 2017). Therefore, a shift towards disease prevention is needed (WHO, 2001). Vaccination is one aspect of prevention, however it is clear that broad vaccination alone appears insufficient to completely alleviate the respiratory disease burden in cattle farms (Windeyer et al., 2012; Dubrovsky et al., 2019). To further reduce its impact additional preventive strategies are essential, such as avoiding exposure to proven risk factors. Besides reduction of infection pressure and improving calf immunity by correct colostrum management (Virtala et al., 1999; Windeyer et al., 2014), in recent years more attention is given to barn climate. Next to thermal comfort, humidity and draught exposure, several air pollutants can be present of which ammonia and bacterial air load are best studied in calves (Lundborg et al., 2005; Lago et al., 2006; Schnyder et al., 2019). For years empirical guidelines and recommendations exist, aiming at a healthy barn climate satisfying the needs for thermal comfort and fresh air supply for calves (CIGR, 1984; DIN 18910, 2017DIN 18910, 2017). Despite these recommendations a serious limitation to elaborate effective preventive strategies, by assuring adequate barn climate, is that it is currently unknown which air quality parameters are actually associated with health parameters of the calves themselves.

Also, for practical and economic reasons, barn air quality parameters that are currently most often evaluated are those that are easily accessible by performing punctual measurements on a single day, such as temperature and relative humidity. As these parameters can show a substantial diurnal variation (Seedorf et al., 1998), continuous 24 -h measurements might improve identification of an unhealthy barn climate. Health effects resulting from exposure to an unhealthy barn climate are upper respiratory tract inflammation and infection, subclinical and clinical pneumonia, all previously included under the term BRD or respiratory disease (Ollivett and Buczinski, 2016). Available studies on the association between barn air quality parameters and respiratory health are limited to clinical signs, using a variety of definitions (Lundborg et al., 2005; Schnyder et al., 2019), with one exception that included thoracic ultrasonography (TUS) (Buczinski et al., 2018). The BRD or respiratory disease concept does not differentiate between airway inflammation and infection nor does it distinguish a pneumonia from upper respiratory tract disease. Today the use of TUS on farm (Ollivett and Buczinski, 2016) combined with validated clinical scoring systems such as the Wisconsin respiratory score (McGuirk and Peek, 2014) and cytology and bacteriology on broncho-alveolar lavage samples offers much more possibilities to differentiate respiratory diseases in term of anatomical localisation (upper or lower respiratory tract) and infectious or non-infectious nature (van Leenen et al., 2020). The primary outcome of interest is lung consolidation, since this parameter has been associated with growth retardation, carcass losses, increased mortality and effects on fertility and longevity (Teixeira et al., 2017; Dunn et al., 2018; Cramer and Ollivett, 2019). To the authors knowledge, no previous work on the association of barn air quality parameters and airway inflammation exists in cattle, despite that airway inflammation of various nature potentially precedes the development of pneumonia, as documented in humans (Siegel and Weiser, 2015). Therefore, the objective of this study was to assess which barn air quality parameters are associated with clinical signs, lung consolidation, airway inflammation and infection of the lower respiratory tract in indoor group-housed calves. This information is essential to identify an unhealthy barn climate and evaluate adaptations to buildings or recommend changes in management practices.

2. Materials and methods

2.1. Study design and sample size calculation

A cross-sectional study was performed with the calf as the experimental unit. With the help of local veterinary practices 60 farms were conveniently selected in Flanders (Belgium) and samples were taken from January to April 2017. Selection of farms was based on willingness to cooperate and informed consent by the farmer. Each farm was visited twice in a 48-h period. On the first day all measurements on indoor air quality were performed and on the second day the calves were sampled to avoid interference with air parameters by animal handling. On each farm the aim was to sample 6–8 calves, aged < 4 months and housed in the same pen or in adjacent pens. If less animals were present all available group-housed calves were sampled. Only calves that had not been treated with antibiotics in the 2 weeks prior to the study were included. All sampling techniques and the study protocol were reviewed by the local ethical committee and permitted under experimental license number EC2016–89.

Sample size was calculated for the multilevel logistic regression with lung consolidation ≥1 cm as outcome variable. Calculations were made to enable detection of a difference of 15 % in the probability of lung consolidation ≥1 cm of depth, between calves exposed to a categorical risk factor (assumed prevalence 30 %) and calves not exposed (assumed prevalence 15 %), accounting for α and β errors of 5% and 20 %, respectively. The estimated sample size was 92 animals per category, resulting in a total of 185 animals. The estimated sample size was adjusted for clustering of calves within a herd, assuming an intraclass correlation of 0.2 and an estimated average number of 7 animals sampled per herd. This resulted in an adjusted sample size of 202 animals per category or a total of 404 animals (Dohoo et al., 2009).

2.2. Farm and housing characteristics

A total of 23 dairy, 23 beef and 14 mixed dairy/beef farms were included in the study. The majority of the farms was medium-sized with 53.3 % (32/60) having less than 100 cows and 36.7 % (22/60) between 100–200 cows in total. In 8.3 % (5/60) of the farms between 200 and 300 cows were present and one farm had > 300 cows. Adult cattle was present in the same building as the calves in 51.7 % (31/60) of the barns. On all farms calves were group-housed with a mean group size of 7 ± 4 calves (range 2–32) per group. The mean pen size was 22.9 ± 15.0 m^2, with a minimum of 4 m^2 and a maximum of 79.7 m^2. On average space per calf was 3.7 ± 2.3 m^2 (range 0.3–14.1). On 33 out of the 60 farms calves were able to have nose-nose contact with calves in the adjacent group pen. Vaccination status of the calves or the adult cattle was not determined.

All herds housed their calves in groups on solid concrete floors bedded with straw, except for one herd where the pen consisted of a slatted floor combined with a straw bedded solid concrete floor. Fresh bedding was added on top of the soiled bedding in all farms. Re-bedding frequencies were once a week on 6.6 % (4/60) of the farms, twice a week on 18.3 % (11/60), three times a week on 30 % (18/60) and ≥4 times a week on 45 % (27/60) of the farms, respectively. A complete cleaning of the pen was performed on 78.3 % (47/60) of the farms, with a frequency of once a week (14.9 %; 7/47), once every two weeks (38.3 %; 18/47), once every month (23.4 %; 11/47) and once every two months (23.4 %; 11/47), respectively. To assess the dryness of the bedding a bedding dryness score was attributed to each farm with score 1 representing a dry, score 2 a moist and score 3 a wet bedding, respectively. Additionally, a nesting score was given to each pen to assess the distribution of fresh bedding, as suggested in a previous study (Lago et al., 2006). Briefly, nesting score 1 was given when calves appeared to lie on top of the bedding with legs exposed, a nesting score 2 was given when the bedding allowed slight nestling and a nesting score 3 was assigned when calves were able to nestle deeply in the bedding.

Calves were fed milk (milk replacer or whole milk with substantial variation between farms) on 53 of the farms, six of these farms supplied milk replacer using an automatic milk feeder. Roughage and/or concentrates were provided on all farms consisting of hay and concentrates (17), silage and concentrates (10), concentrates (8), silage (9), silage and hay (9), silage and straw (2) and straw and concentrates (5).

A total of 38.3 % (23/60) of the calf barns was open on one side and 36.7 % (22/60) was open on two sides, 25 % (15/60) of the barns were completely enclosed. Natural ventilation was used in the majority of the barns, except for five farms which used mechanical ventilation systems (3 positive pressure tube systems and 2 negative pressure systems). Most of the naturally ventilated farms (43.6 %; 24/55) were oriented with their long axis perpendicular to the prevailing wind direction, which is south-west in the studied area. Pen partitioning that was not completely solid and allowed air circulation between pens, was present in 66.7 % (40/60) of the farms. Protective measures at the inlet to prevent high air velocity at calf level were present in 53.3 % (32/60) of the farms and consisted of curtains in 50 % (16/32), space boarding in 31.3 % (10/32), and a solid protective cover for shelter at calf height in 18.7 % (6/32) of the farms. The average air volume per calf was 24.8 ± 50.9 m^3 (range 1.9–377.2).

2.3. Measurement of air quality parameters

Measurements of indoor air quality parameters were performed during the day of the first visit. As the study objective focused on indoor air quality parameters associated with calf respiratory health, measurements were performed at calf breathing level. Sampling equipment was placed in the pen where all sampled calves were housed or in the pen that housed the majority of the calves in case the adjacent pen also needed to be sampled to obtain the pre-set sample size of 6 calves on each farm.

Continuous 24 -h measurements were performed for temperature and relative humidity on all 60 farms and for ammonia concentration on 27 of the farms. Ammonia was measured continuously on half of sampled farms because only one sampling device could be used that was rotated between farms. The sampling equipment used for these continuous measurements was placed in the left front corner of the pen with the sensors placed 50 cm above ground level which corresponds with the breathing level of the calves. To avoid damage of the equipment by the animals all sampling devices were placed in a custom made mesh wired box (100 × 69.5 cm), with a mesh size of 4.2 × 8.8 cm, secured to the headlocks or feed barrier.

Temperature and relative humidity were measured and logged with a one-minute measuring interval, using a mobile weather housing aerosol spectrometer (Grimm 1.109, Grimm Aerosol Technik GmbH & Co. KG, Ainring, Germany) equipped with a meteorological sensor (1.153FH) for temperature (range (R): 0.3–80 °C, accuracy ± 0.3 K) and relative humidity (R: 0 − 100%, accuracy ± 1%). Mean temperature and relative humidity and maximum and minimum values over the 24 -h measuring period were calculated.

Ammonia concentrations were measured in two different ways using one-time measurements and a continuous measuring device. Continuous measurements for ammonia were carried out using a pumped handheld device with electrochemical sensor (R: 0 − 100 ppm, accuracy ± 10 %) (MulitRAE lite, RAE Benelux, Hoogstraten, Belgium) attached to the side of the protective mesh wired box. Measurements were carried with a 30 s measuring interval and mean 24-h ammonia concentration was calculated.

Punctual one-time measurements detecting air velocity and measuring CO_2 and ammonia concentrations were performed on each farm during the morning of the first visit in the right front, middle and left back of the pen, respectively. A punctual measurement for the concentration of CO_2 or ammonia was obtained during a one-minute measuring period and was defined as the concentration at which the sensor reached equilibrium. Air velocity was measured using a handheld thermal anemometer (R: 0 − 30 m/s, accuracy 0.06 m/s) (Airflow™ TA45, TSI Inc, Shoreview, MN). A punctual measurement value of air velocity was defined as the speed at which the sensor indicated a steady state velocity during the 30 s measuring period. Maximal air speed measured in any of the three sampling places of the pen was used for analysis. Levels of CO_2 (R: 0 − 50000 ppm, accuracy ± 5.0 %) and NH_3 (R: 0 − 500 ppm) in each pen were measured using an aspirated handheld monitor (IBRID™MX6, Industrial Scientific, Pittsburgh, PA). All used instruments were calibrated by the manufacturer before use.

Microbiological air quality in the pen was measured by sampling one litre of air onto a Columbia blood agar plate with 5% sheep blood (Oxoid™, Hampshire, UK) using an impaction air sampler (MAS-100 Eco®, Merck KGaA, Darmstadt, Germany). To avoid interference of bacteria coming from the calf or the stable bedding the sampler was placed with the inlet faced to the roof and placed in the middle of the box on a block with a height of 30 cm above ground level. To correct for the background bacterial contamination in the air, on each farm an outdoor baseline measurement was performed with a minimal distance of 5 m from the barn, using the same sampling procedure. Agar plates were incubated for 24 h at 35 °C without CO_2 and colony-forming units were counted manually. To obtain the final pen bacterial air load baseline measurements were subtracted from pen measurements.

2.4. Clinical examination, thoracic ultrasonography and broncho-alveolar lavage

During the second day of the farm visit, all examinations of the calves were performed after dismanteling all sampling equipment to avoid interference of movement of the calves with the climatic data. Clinical examination of the calves was performed and animals were considered healthy or diseased based on the Wisconsin calf respiratory score (McGuirk and Peek, 2014), with calves scoring ≥5 considered diseased. Thoracic ultrasonorgraphy was performed including bilateral examination of the cranial lung lobes, as previously described (Pardon, 2019). A portable ultrasound with a linear probe with a frequency of 7.5 MHz set at a depth of 8 cm (Tringa Linear Vet®, Esaote, the Netherlands) was used with 70 % isopropylalcohol as a transducing agent. Presence of lung consolidation was documented according to location (dorsal/ventral ; left/right) and depth in centimetres measured in a dorso-ventral plane.

After clinical and thoracic ultrasonographic examination a small-volume (0.6 mL/kg body weight) non-endoscopic broncho-alveolar lavage (nBAL) was performed in standing unsedated calves, to collect broncho-alveolar fluid (BALf) for bacteriological and cytological analysis, as described previously (Van Driessche et al., 2017). Cytological examination of BALf consisted of manual determination of total nucleated cell count (TNCC) using a haemocytometer followed by calculation of the differential cell count by counting 400 cells on cytospin preparations, as described previously (van Leenen et al., 2020).

Bacterial culture of BALf for *Pasteurellaceae* and *Mycoplasma bovis* was done on Columbia blood agar and pleuro pneumonia-like organism agar (PPLO), respectively, as more extensively described elsewhere (Van Driessche et al., 2017). Species confirmation of *Pasteurellaceae* and *M. bovis* was performed using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Brüker Daltonik GmbH, Bremen, Germany). Bacterial cultures were interpreted according to isolation of target bacteria, commonly associated with BRD in calves, namely: *Pasteurella multocida, Mannheimia haemolytica, Histophilus somni* and *Mycoplasma bovis.* Culture results were considered negative for the target bacteria if growth was absent after an incubation period for 48 h for *Pasteurellaceae* and 5 days for *M.* bovis, or if contaminants or multiple bacterial colonies with different morphology were present on the agar plate (polymicrobial) and no target bacteria could be subjected to subculture for further identification. Bacterial cultures were considered positive if a dominant or pure culture result was obtained for the target bacteria (Van Driessche et al., 2017). All examinations, sampling procedures and sample analyses were performed by the same veterinarian.

2.5. Statistical analyses

Data were analysed in two different ways, first linear regression and

mixed model analyses were performed. Second, a cluster analysis of the indoor air quality parameters was carried out.

2.6. Linear regression and mixed models

Analyses were performed using SAS® version 9.4 (SAS Institute Inc., Cary, N.C.) for Windows. The experimental unit was the calf. Four groups of outcomes were tested: clinical signs (1 binary outcome), lung consolidation (3 binary outcomes), airway inflammation (5 continuous outcomes and 2 binary outcomes) and lower respiratory tract infection (4 binary outcomes). For binary outcomes 0 represents a negative and 1 represents a positive outcome. The presence of clinical signs was represented by a positive Wisconsin respiratory score, analysed as the binary outcome variable WI-score (0/1). Lung consolidations were analysed as three binary outcomes: lesion depth ≥1 cm (0/1), ≥3 cm (0/1), and ≥6 cm (0/1), respectively. Lesion depth was measured on ultrasonographic images, if bilateral lesions were present the lesion of maximum depth was used to assign a score to the calf. For airway inflammation no clear cut-off values are present to date defining different types of inflammatory BALf profiles in calves. Therefore, airway inflammation was analysed as a group of continuous outcome variables (TNCC, neutrophil percentage, macrophage percentage, lymphocyte percentage, epithelial cell percentage, basophil percentage and eosinophil percentage). These continuous outcome variables were checked for a normal distribution and log + 1 transformed when required. Log transformation was needed for TNCC, lymphocyte percentage and epithelial cell percentage. Neutrophil and macrophage percentage were normally distributed. For eosinophil and basophil percentage no transformation to a normal distribution was possible, therefore these outcomes were analysed as binary outcomes. Samples containing > 1% eosinophils were considered positive (increased). For basophils, a sample was considered positive if any of these cells were seen in the 400 cells counted (Meyer, 2007). A lower respiratory tract infection was deemed to be present in the calf when at least one of the pathogens *Pasteurella multocida, Mannheimia haemolytica, Histophilus somni* or *Mycoplasma bovis* was isolated from BALf in pure or dominant culture. This was represented by the binary outcome variables *P. multocida* isolation (0/1), *M. haemolytica* isolation (0/1), *H. somni* isolation (0/1) and *M. bovis* isolation (0/1).

Predictors were continuous measurements of temperature, relative humidity and ammonia and punctual measurements of air velocity, ammonia, CO_2 and bacterial air load. For temperature and relative humidity the 24 -h average was tested as continuous value. From the average 24 -h ammonia concentration in ppm as original continuous value a derived variable was created for analytical purposes. Based on one of the parameters of interest, ultrasonographic consolidation ≥1 cm in depth, a new predictor was created using the maximum ammonia concentration measured over a 24-h period. To determine the optimal cut-off value for the maximum ammonia concentration, to predict the presence of ultrasonograpic consolidation ≥ 1 cm in depth with highest sensitivity and specificity, a receiver operating characteristics (ROC) curve with the Youden's index was used. Using this approach the derived variable time ammonia > 4 ppm (hours) was created as a derived continuous predictor variable.

Four punctual measurements were tested: air velocity, ammonia in the front of the pen, CO_2 in the front of the pen and total bacterial air load (cfu/m^3). Ammonia concentration, CO_2 concentration and total bacterial air load were analysed as continuous variables. Since obtained values of the punctual air velocity were very low and close to the detection limit in a number of barns, this parameter was analysed as a derived binary variable. Based on the maximum air velocity measured at any of the three places in the pen (front, middle or back) a ROC curve with the Youden's index was created to determine the optimal maximum air velocity cut-off value with highest sensitivity and specificity to predict the presence of an ultrasonographic consolidation of ≥1 cm in depth. The binary variable air velocity > 0.8 m/s (0/1) was created after this procedure.

Five multivariable linear regression models (PROC MIXED) were made to determine the association of TNCC, neutrophil percentage, macrophage percentage, lymphocyte percentage, and percentage of epithelial cells, as outcome variables representing airway inflammation, with the seven predictors mentioned above. In each model herd was added as a random effect to account for clustering of calves within a herd. In a first step the association of the different predictors with the outcome variable was tested univariably. All parameters with $P < 0.20$ were withheld for the next step. Pearsons and Spearman correlation were determined, and of predictors correlated over 0.6, only the most significant one was withheld from the modelling process. The regression model was built stepwise backwards, gradually excluding not significant variables. Significance level was set at $\alpha = 0.05$ with a 95 % confidence interval. Pairwise comparisons between different categories of significant effects were made using Bonferroni corrections. Biologically plausible interactions between significant main effects were tested. Model fit was assessed by visual inspection of residual plots and normality testing of residuals.

The association of the seven predictors with the binary outcome variables Wisconsin respiratory score (representing clinical signs), ultrasonographic consolidation ≥1 cm, ultrasonographic consolidation ≥3 cm, ultrasonographic consolidation ≥6 cm, eosinophils, basophils (representing airway inflammation) and *P. multocida* isolation, *M. haemolytica* isolation, *H. somni* isolation and *M. bovis* isolation (representing lower respiratory tract infection) was determined using a generalized linear mixed model (PROC GLIMMIX) with binomial distribution and logit link function with Wald's statistics for type 3 contrasts. Herd was added as a random factor to account for clustering of calves within a herd. First, the same predictors as mentioned above were tested in univariable analysis. The same variable selection criteria as mentioned above were used for the further model building procedure. Model fit was evaluated using the Hosmer-Lemeshow goodness-of-fit test for logistic models. All possible and biologically relevant interactions between main effects were tested.

2.7. Cluster analysis

A cluster analysis of indoor air quality parameters was performed to identify barns with similar climatic conditions (barn climate clusters) using SPSS® version 25.0 (IBM®, Armonk, NY, 2019). K-means clustering was conducted to determine the centroid with a minimized within cluster sum of squared Euclidean distance among each observation. Using this clustering method farms were grouped based on standardized values of average 24 -h temperature, average 24 -h relative humidity, punctual NH_3 and CO_2 concentration in front of the pen, bacterial air load and the maximum air velocity. The number of clusters was determined by the Elbow method (scree plot), which plots the within-cluster sum of square according to the number of clusters k. This resulted in a number of four clusters: 1 (n = 19), 2 (n = 8), 3 (n = 25), 4 (n = 3). Clusters were labelled based on the dominant factors being above average measurements of temperature and below average measurements of relative humidity as indicators of a dry and warm climate, above average measurements of ammonia, CO_2 and bacterial air load as signs of an under-ventilated stable with accumulation of air pollutants. Above average maximum air velocity is used as indicator for the presence of draught. To examine the associations between barn climate clusters and clinical signs and lung consolidation a single factor logistic regression was performed for WI-score, as representing variable for clinical signs, and lung consolidation ≥1 cm, ≥3 cm and ≥6 cm, respectively. To determine associations between barn climate clusters and airway inflammation a single factor linear and logistic regression was performed for TNCC, neutrophil percentage, macrophage percentage, lymphocyte percentage and epithelial cell percentage as continuous variables and eosinophils and basophils a binary variables, respectively. To determine associations between barn climate clusters and

lower respiratory tract infection a single factor logistic regression was performed for the 4 outcome variables of interest: *P. multocida* positive, *M. haemolytica* positive, *H. somni* positive and *M. bovis* positive. Herd was added as random factor to account for clustering of calves within a herd. Model building and fit evaluation was as described above.

3. Results

3.1. Calf characteristics

A total of 428 calves included in the study, 4.0 % (17/428) were < 4 weeks old, 35.7 % (153/428) 4–8 weeks old and 60.3 % (258/428) were 8 weeks and older. The Holstein-Friesian breed (dairy) was represented in 49.8 % (213/428) of the calves, the Belgian Blue breed (beef) in 45.1 % (193/428) and 5.1 % (22/428) of the calves was of a mixed breed (dairy and beef crossbreeds). The average number of eligible calves present on each farm was 6 ± 3 (R = 2–22) and on average 7 ± 2 (R = 2–10) calves per farm were sampled.

3.2. Indoor climate and barn measurements

Temperature and relative humidity was measured on 58 and 57 farms, respectively. Missing values were due to technical failure of equipment or human error. Continuous measurements of ammonia were performed on 27 farms. Punctual measurements of air velocity, CO_2, ammonia and bacterial air load were performed on 60 farms. Results of these measurements on calf breathing level are presented in Table 1. Bacterial air load concentration was corrected for outdoor baseline bacterial concentrations which were on average 63.3 cfu/m^3 (SD 92.6; min 0; max 411). On 33.3 % (9/27) of the farms where continuous ammonia measurements were performed, the maximum NH_3 concentration exceeded the 4 ppm threshold. The average time NH_3 > 4 ppm was 0.51 ± 1.47 h (range 0–6.3 hour). On 5% (3/60) of the farms air velocities > 0.8 m/s could be measured in a minimum of one of the three measurement points in the pen.

Continuous measurements of minimum, maximum and average temperature were positively correlated (Range (R) = 0.80 – 0.95; $P < 0.01$), the same positive correlations were found between minimum, maximum and average relative humidity measurements over a 24-h measuring period (R = 0.56 – 0.91; $P < 0.01$). Temperature and relative humidity measurements (minimum, maximum and average) were negatively correlated (R ranging from -0.21 – -0.66; $P < 0.01$).

Continuous ammonia measurements, punctual ammonia measurements, air velocity, CO_2 and bacterial air load were not correlated with any of the other variables measured. Punctual measurements of NH_3 (R = 0.90 – 0.93; $P < 0.01$) and CO_2 (R = 0.53 – 0.71; $P < 0.01$) in different places of the pen were mutually positively correlated.

A bedding dryness score of 1 was assigned on 36.7 % (22/60) of the farms, score 2 on 40 % (24/60) and score 3 on 23.3 % (14/60) of the farms, respectively. A nesting score of 1 was given on 76.7 % (46/60), a score of 2 on 20 % (12/60) and a score of 3 on 3.3 % (2/60) of the farms, respectively. Bedding dryness score or nesting score were not associated with any of the air quality parameters studied.

3.3. Clinical signs, ultrasonographic lesions, BALf cytology and bacteriology

A Wisconsin score ≥5 was observed in 20.2 % (86/425) of the calves. Ultrasonographic consolidations of ≥1 cm, ≥3 cm and ≥6 cm were present in 41.1 % (176/428), 27.1 % (116/428) and 16.1 % (69/428) of the calves, respectively.

Within-herd prevalence of lung consolidation was 40.8 % (SD 29.7, R = 0−100) for lung consolidation ≥1 cm in depth, 28.7 % (SD 26.9, R = 0−100) for ≥3 cm in depth and 18.5 % (SD 26.9, R = 0−100) for ≥6 cm in depth.

Total nucleated cell count (TNCC) could be determined in 404 calves and complete cytological differential cell count profiles of BALf were available for 80.1 % (343/428) of the calves. Reasons for loss of samples were staining errors and artefacts hindering a reliable interpretation of cell types. Mean BALf TNCC concentration was 1.8 × 10^9 cells/L (SD 1.7, R 0–13.7). Differential cell counts are represented in Table 2. Basophils were present in BALf of 10.2 % (35/343) of the calves and eosinophilia (> 1% eosinophils) was noted in 12.8 % (44/341). BALf was positive for *P. multocida* in 31.8 % (136/428), for *M. haemolytica* in 14.5 % (62/428), for *H. somni* in 3.7 % (16/428) and for *M. bovis* in 2.8 % (12/428) of the calves, respectively.

3.4. Associations of indoor air quality parameters with WI-score and ultrasonographic findings

Mixed model analysis was performed on a dataset of 394 calves for Wisconsin respiratory score. The results of this analysis are presented in Table 3.

Continuous ammonia measurements were not associated with ultrasonographic findings. The ROC analysis for maximum 24 -h

Table 1
Indoor air quality parameters, measured at calf breathing level, in group-housing pens (January to April 2017, Belgium).

Parameter	Number of farms	Mean ± SD	Range (min-max)
Continuous measurements[a]			
Minimal pen temperature (°C)	58	10.6 ± 4.2	2.5 – 20.0
Maximal pen temperature (°C)	58	17.5 ± 4.9	9.2 – 28.1
Average pen temperature (°C)	58	14.2 ± 4.4	5.5 – 23.9
Difference pen temperature (°C)	58	7.0 ± 2.8	2.2 – 14.7
Minimal pen relative humidity (%)	57	54.6 ± 12.6	30.8 – 86.2
Maximal pen relative humidity (%)	57	81.8 ± 8.9	46.7 – 95.4
Average pen relative humidity (%)	57	68.8 ± 8.9	52.2 – 91.6
Difference relative humidity (%)	57	27.6 ± 11.5	0 – 48.1
Minimal pen ammonia (ppm)	27	0.1 ± 0.3	0 – 1.0
Maximal pen ammonia (ppm)	27	4.2 ± 2.1	1 – 10.0
Average pen ammonia (ppm)	27	1.7 ± 0.9	0 – 4.0
Punctual measurements			
Maximal air velocity pen (m/s)	60	0.1 ± 0.3	0 – 2.0
CO_2 front pen (ppm)	60	1758.5 ± 1743.0	0 – 7800
CO_2 middle pen (ppm)	60	1926.7 ± 1526.8	0 – 7000
CO_2 back pen (ppm)	60	1890.0 ± 1676.9	0 – 8000
Ammonia front pen (ppm)	60	2.23 ± 1.23	0 – 6.0
Ammonia middle pen (ppm)	60	2.32 ± 1.23	1 – 7.0
Ammonia back pen (ppm)	60	2.37 ± 1.19	1 – 7.0
Bacterial air load (cfu/m^3)	60	247002 ± 113307	28000 - 400000

[a] Continuous measurements performed over a 24-h period. SD = standard deviation.

Table 2
Differential cell counts in broncho-alveolar lavage fluid of 343 indoor group-housed weaned and pre-weaned calves (January to April 2017, Belgium).

Cell type	Mean (%) ± SD	Range (min-max)
Neutrophils	37.2 ± 23.7	0 – 97.4
Macrophages	42.4 ± 18.8	2.4 – 92.3
Lymphocytes	5.2 ± 5.0	0 – 45.8
Eosinophils	0.3 ± 0.8	0 – 9.1
Basophils	0 ± 0.1	0 – 0.7
Epithelial cells	14.8 ± 13.0	0 – 95.9

Table 3
Multivariable logistic regression model for the association of indoor air quality parameters with Wisconsin respiratory score (≥5) in indoor group-housed calves (n = 388).

Variable	Regression coefficient β (SE)	OR	95 % CI	*P*-value
Intercept	−2.66 (0.74)			< 0.0001
Average pen relative humidity (%)	0.04 (0.018)	1.04	1.01 – 1.08	0.03

SE = standard error, OR = odds ratio, CI = confidence interval.

ammonia concentration demonstrated that the optimal cut-off (area under the curve = 0.58; sensitivity = 45.0 %; specificity = 75.9 %) to predict the presence of an ultrasonographic consolidation with a depth of ≥1 cm was 4 ppm. Using this derived variable, in the multivariable model the time (hour) that ammonia exceeded the 4 ppm cut-off ppm was associated with ultrasonographic consolidation of ≥1 cm in depth (Table 4). Punctual ammonia measurements were not associated with ultrasonographic consolidation of any depth.

To explore the associations of air velocity with ultrasonographic consolidation a ROC analysis was performed demonstrating that the optimal cut-off (area under the curve = 0.508; sensitivity = 10.2 %; specificity = 98.4 %) to predict the presence of an ultrasonographic consolidation with a depth of ≥1 cm was an air velocity of 0.8 m/s. In the multivariable model, the presence of an air velocity > 0.8 m/s on any of the three places in the pen was associated with ultrasonographic consolidation ≥3 cm and ≥6, as is shown in Table 4. Of all other indoor climate parameters only pen temperature was associated with consolidation of all depths (Table 4).

3.5. Associations of barn air quality parameters with BALf cellular variables and bacterial culture

The multivariable models for TNCC, neutrophil percentage, lymphocyte percentage and epithelial cell percentage are presented in Table 5. Increases in ammonia increased TNCC and percentage of epithelial cells. The odds for having > 1% basophils increased in more moist environments (OR = 1.1 for each increase in average relative humidity by 1%; 95 % CI = 1.04–1.13; *P* = 0.0001). No associations of any of the air quality parameters with BALf neutrophil, macrophage or lymphocyte percentage could be found. None of the bacterial respiratory pathogens isolated from BALf were associated with the air quality parameters studied.

Table 5
Linear regression models for the association of broncho-alveolar lavage fluid characteristics with indoor air quality parameters in indoor group-housed calves (January to April 2017, Belgium).

Variable		Regression coefficient β (SE[b])	*P*-value
TNCC (cells x 10^9/L) (n = 404)			
Intercept		1.09 (0.11)	< 0.0001
Ammonia front pen (ppm)		−0.04 (0.02)	0.03
Air velocity > 0.8 m/s	Absent		
	Present	−0.30 (0.11)	0.014
Epithelial cells (%) (n = 311)			
Intercept		7.4 (0.14)	< 0.001
Ammonia front pen (ppm)		0.14 (0.05)	0.01

TNCC = total nucleated cell count, SE = standard error, OR = odds ratio, CI = confidence interval.

3.6. Barn climate cluster analysis

Four distinct climatic clusters could be identified on farm level based on average pen temperature and relative humidity, maximal air velocity, NH_3 and CO_2 concentration and bacterial air load, which are displayed in Fig. 1. All evaluated parameters contributed significantly to the clusters. Barns in cluster 1 were characterised by a warm and dry climate with ammonia accumulation (n = 19). Cluster 2 represented under-ventilated barns with accumulation of air pollutants (NH_3, CO_2) and high bacterial air load (n = 8). Temperature and relative humidity values were close to average in cluster 3, furthermore barns in this cluster displayed low concentrations of NH_3, CO_2 and bacteria in the air combined with low air velocity. Therefore, cluster 3 was labelled as a presumably normal indoor climate characterised by sufficient ventilation (n = 25). Cluster 4 was labelled as the draught cluster, characterised by high air velocity (n = 3).

Table 4
Multivariable logistic regression model for the association of indoor air quality parameters with ultrasonographic consolidation of variable depth in indoor group-housed calves (January to April 2017, Belgium).

Variable		Regression coefficient β (SE)	OR	95 % CI	*P*-value
Ultrasononographic consolidation ≥1 cm (n = 184)					
Intercept		−3.66 (1.12)			0.0003
Average pen temperature (°C)		0.19 (0.068)	1.22	1.06 – 1.40	0.005
Time ammonia > 4 ppm (hour)		0.57 (0.28)	1.73	1.02 – 3.07	0.04
Ultrasononographic consolidation ≥3 cm (n = 414)					
Intercept		−1.5 (1.04)			0.16
Average pen temperature (°C)		0.14 (0.05)	1.2	1.06 – 1.26	0.002
Air velocity > 0.8 m/s	Absent	Referent			
	Present	1.9 (0.9)	6.8	1.23 – 38.5	0.03
Ultrasononographic consolidation ≥6 cm (n = 414)					
Intercept		−4.8 (1.8)			0.0009
Average pen temperature (°C)		0.32 (0.08)	1.4	1.17 – 1.63	0.0002
Air velocity > 0.8 m/s	Absent	Referent			
	Present	2.8 (1.3)	15.9	1.2 – 200	0.03

SE = standard error, OR = odds ratio, CI = confidence interval.

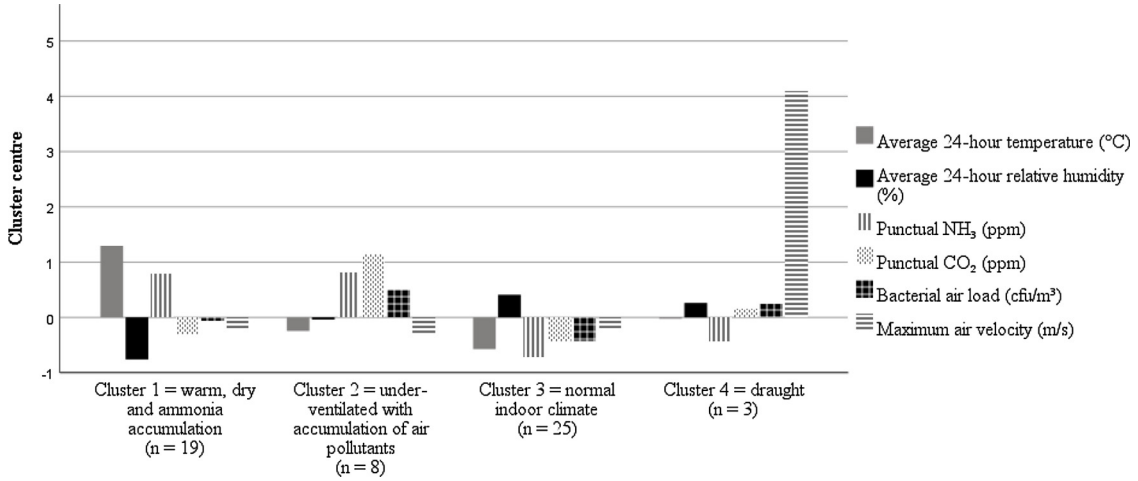

Fig. 1. Climate clusters of 55 naturally ventilated barns used for group-housing of (pre-)weaned calves in Belgium.

3.7. Associations of barn climate clusters with WI-score, ultrasonographic findings, BALf cytology and bacteriology

The warm, dry and ammonia cluster (cluster 1) consisted of 118 calves, the under-ventilated cluster (cluster 2) of 65, the presumably normal climate cluster (cluster 3) of 187, and the draught cluster (cluster 4) of 22 calves, respectively. No associations of barn climate clusters with WI-score could be evidenced. Lung consolidations of all depths were associated with barn climate clusters (Fig. 2). The percentage of calves with an ultrasonographic consolidation of ≥1 cm was higher in the warm, dry and ammonia accumulation cluster (54.2 %) compared to the presumably normal climate cluster (31.6 %) (P = 0.02). Additionally, a significant difference was found for the presence of lung consolidation with a depth of ≥1 cm between the under-ventilated and draught cluster (32.3 % in the under-ventilated cluster versus 81.8 % in the draught cluster, P = 0.026) and for the normal versus the draught cluster (31.6 % in the normal cluster versus 81.8 % in the draught cluster, P = 0.0092).

Ultrasonographic consolidations with a depth of ≥3 cm were more frequently present in the warm, dry and ammonia accumulation cluster (38.1 %) compared to the normal climate cluster (18.2 %) (P = 0.04). The same results were found for ultrasonographic consolidation ≥6 cm in depth, which was 31.4 % in the warm, dry and ammonia accumulation cluster compared to 9.1 % in the presumably normal climate cluster (P = 0.01). Cytological findings in BALf stratified by climate cluster are displayed in Table 6. Of all cytological parameters only BALf epithelial cell percentage was significantly higher in the warm, dry and ammonia accumulation cluster (16.4 ± 0.8 %) compared to the presumably normal barn climate cluster (12.8 ± 1.0 %) (P = 0.01).

No significant associations were present for bacterial isolation from BALf and the four different climate clusters.

4. Discussion

For decades the role of barn climate in the prevention of BRD has been recognized (Ames, 1997; Lago et al., 2006; Lundborg et al., 2005; Schnyder et al., 2019). However the specific parameters that should be monitored or controlled remain a subject of debate. In an attempt to clarify some of these issues we performed a cross-sectional study on both dairy and beef farms representative for the current housing conditions of pre-weaned and weaned calves in medium-size farms in different European countries.

Our study showed that temperature, relative humidity, air velocity and ammonia concentration are associated with lung consolidation and

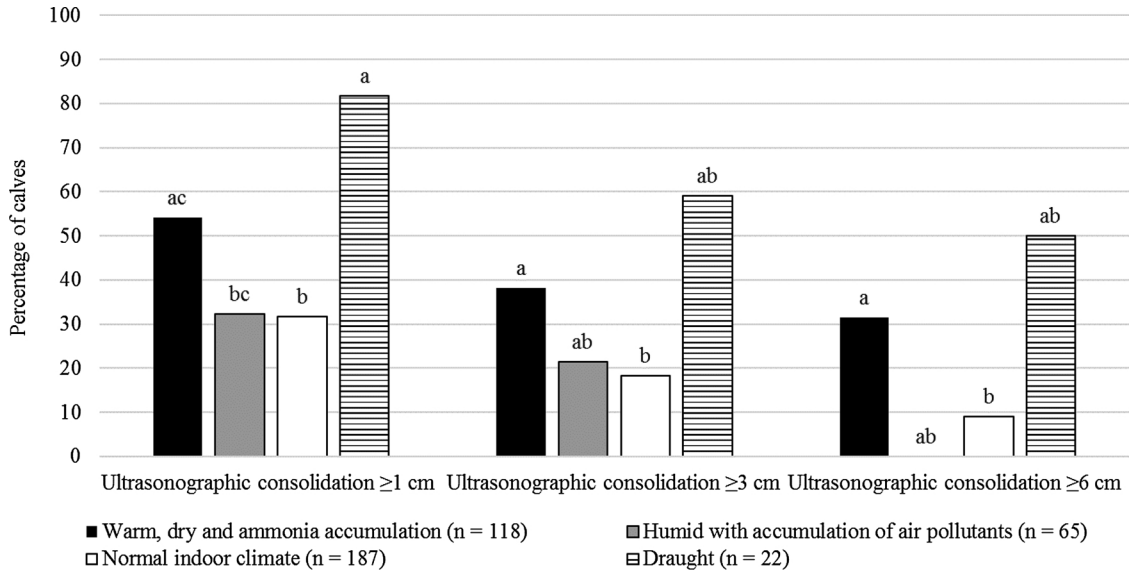

Fig. 2. Ultrasonographic findings in 392 indoor group-housed (pre-) weaned calves stratified by barn climate clusters.
Different letters indicate significant differences between groups (P < 0.05).

Table 6
Cytological findings in broncho-alveolar lavage fluid of 392 indoor group-housed pre-weaned calves, stratified by barn climate cluster (January to April 2017, Belgium).

	Warm, dry and ammonia accumulation (n = 118)	Humid with accumulation of air pollutants (n = 65)	Normal indoor climate (n = 187)	Draught (n = 22)
	Mean ± SD (min – max)	Mean ± SD (min – max)	Mean ± SD (min – max)	Mean ± SD (min – max)
BALf TNCC (cells x 10^9 /L)	1.5 ± 1.2 (0.02–5.9)	1.9 ± 1.7 (0.02–8.2)	2.0 ± 2.1 (0.04–13.8)	1.0 ± 0.7 (0.07–2.4)
BALf neutrophils (%)	35.0 ± 19.9 (0.5–84.3)	39.3 ± 27.7 (11.1–97.4)	40.7 ± 25.6 (0–89.4)	36.2 ± 18.6 (3.3–66.2)
BALf macrophages (%)	42.0 ± 15.9 (9.7–77.6)	39.6 ± 20.5 (2.4–82.4)	41.2 ± 19.8 (8.6–89.6)	43.7 ± 17.2 (14.5–75.4)
BALf lymphocytes (%)	5.8 ± 4.0 (0.5–24.3)	4.9 ± 4.9 (0.2–20.2)	5.0 ± 6.0 (0–45.8)	5.9 ± 4.3 (0.2–18.8)
BALf epithelial cells (%)	16.4 ± 8.6 [a] (0.7–39.0)	15.9 ± 17.8 [ab] (0–95.9)	12.8 ± 12.1 [b] (0–72.1)	13.9 ± 9.8 [ab] (0.4–41.6)

Different letters indicate significant differences between groups ($P < 0.05$).

BALf cellular composition and could therefore be useful parameters in the evaluation of calf barn climate. Also, the importance of evaluating indoor climate by combined analyses of a variety of parameters was illustrated, enabling identification of two types of indoor climate associated with respiratory disease. First, barns likely coping with insufficient ventilation were identified, characterised by a warm and dry indoor climate with accumulation of ammonia. Secondly, barns with possible problems due to cold stress induced by excessive ventilation or draught exposure were found, characterised by presence of a high air velocity. The different parameters associated with respiratory disease in our study will be discussed below.

First, the effects of temperature. The hypothesis was that low temperatures were associated with respiratory disease in calves, since recommended temperatures range from 16 − 20 °C for calves less than 60 kg of bodyweight and 10 − 20 °C for calves of 60 − 150 kg (DIN 18910, 2017DIN 18910, 2017). Our results indicate that temperature indeed plays a role, but in the opposite direction with increasing pen temperatures being associated with ultrasonographic lesions. Additionally, the percentage of calves with ultrasonographic consolidations of all depths was higher in the warm, dry and ammonia accumulation cluster compared to the presumably normal climate cluster. Our results resemble recent studies indicating that heat stress or warm and dry environmental conditions are associated with increased BRD incidence (Louie et al., 2018) and mortality in pre-weaned calves (Egberts et al., 2019). These findings suggest that calves are tolerant and adaptable to moderately cold ambient temperatures when provided with a high plane of nutrition and kept in a dry bedded environment, as stated by others (Rawson et al., 1989; Lago et al., 2006; Nonnecke et al., 2009). This is in contrast to the relation between exposure to cold ambient temperatures, and decreasing humidity, and increased occurrence of respiratory infections in humans (Mäkinen et al., 2009). However, studies in humans suggest that there is a certain lag period between changes in temperature or humidity levels and disease (Mourtzoukou and Falagas, 2007; Mäkinen et al., 2009). Since we only measured temperature and humidity levels during a relatively short 24 -h period and lesions found in the calves could be related to climatic conditions days or weeks before our visit these relations could not be evidenced in our study. Furthermore, despite parallels with existing literature it should be noted that temperature ranges in our study were very moderate, typical for European weather conditions that are partially reflected in the indoor climate in naturally ventilated barns (Seedorf et al., 1998). Extremely cold or high temperatures were not measured, although in several barns temperatures below recommended minimal temperature of 10 °C for the age and weight categories studied (CIGR, 1984) were noted. Finally, temperature effects might be more indirect, for example by increasing ammonia production as is often suggested (Herbut and Angrecka, 2014). However, these correlations could not be reproduced with our data, resembling other studies where these correlations could only be found in poultry and pig housings, but not in cattle barns (Seedorf and Hartung, 1999; Kaufman et al., 2015). Differences in floor types, floor bedding, ventilation system and air volume between housing modalities of different species could explain these findings.

Second, the effects of relative humidity. Recommendations for relative humidity range from 60 to 80% in mechanically ventilated stables whereas 40–70 % should suffice in heated stables (DIN 18910, 2017DIN 18910, 2017). For naturally ventilated stables no recommendations considering this parameter are available. Average relative humidity was the only parameter significantly linked with Wisconsin respiratory score in the multivariable models of our study. Calves housed in a barn with a higher average pen relative humidity over a 24 -h period, had increased odds of being WI-score positive. An increased relative humidity could be resulting from wet bedding conditions in the pen, which could also augment the effects of cold and increased wind velocity on calf level, possibly explaining the associations with the WI-score. Increased 24 -h relative humidity was also associated with an increased odds of finding basophils in BALf. A more humid indoor climate could enhance growth of moulds and fungi. Inhalation of aerosols containing spores or fungal parts could initiate an inflammatory or allergic reaction, characterised by presence of basophils in BALf, as has been shown in horses (Beeler-Marfisi et al., 2010). Possibly, the effects of relative humidity are indirect and complex by hampering or promoting survival of respiratory pathogens, depending on their type and characteristics (Ames, 1997). Furthermore, relative humidity and temperature are biologically interrelated. If the water content of air stays the same and temperature increases, the relative humidity will decrease because hot air can hold more water compared to colder air. The impact of relative humidity on respiratory tract immunity, and its influence on survival of different respiratory pathogens needs to be further investigated.

Third, the effects of ammonia. The presence of mild ultrasonographic lesions (≥1 cm consolidation) was associated with prolonged ammonia exposure and calves housed in the warm, dry and high ammonia climate showed an increased percentage of lung consolidation compared to the presumably normal climate cluster. Remarkably, only exposure to concentrations above a certain threshold, in this case 4 ppm, was linked with pneumonia. These results could possibly indicate that chronic exposure to low concentrations is involved in the BRD complex, and it would be advisory to measure concentrations continuously over a period of time. However, punctual ammonia concentrations could render important information when combined with other climate measurements at the same time, such as relative humidity and temperature, to distinguish climatic clusters which could provide information on the complex interaction of indoor climate and respiratory disease. Albeit that measured ammonia concentrations in our study were comparable to other studies in both Europe (Seedorf and Hartung, 1999) and other parts of the world (Lago et al., 2006; Kaufman et al., 2015; Buczinski et al., 2018), it should be noted that during our 24 -h measuring period overall very low values, close to the detection limit of the used devices, were measured. Therefore, results should be interpreted with caution. We suggest that future studies consider use of more sensitive analytical measurement methods to evaluate ammonia concentrations in calf housings. Furthermore, since measurements were only performed for a short 24 -h period or as a one-

time measurement no information on climatic conditions in the period preceding our visit was available. Possibly calves were exposed to higher ammonia concentrations in the period before our visit. This could have resulted in lesions that were detected during our visit, since ultrasonographic lesions can also represent an earlier episode of respiratory disease. Notwithstanding, although ammonia concentrations are substantially lower than the recommended maximum value of 20 ppm (CIGR, 1984), a handful of other studies also showed associations of disease with ammonia concentrations lower than this documented threshold value. Associations with concentrations of ≥10 ppm with increased antimicrobial use in veal calves are demonstrated (Schnyder et al., 2019). Others found a counterintuitive protective effect of a high ammonia concentration (≥6 ppm) on the risk of respiratory disease (Lundborg et al., 2005). However, the latter study classified calves as healthy or diseased based on thoracic auscultation and clinical scoring, examination methods which are less accurate compared to thoracic ultrasonography (Buczinski et al., 2015). Besides associations with ultrasonographic lesions our study was the first to demonstrate a positive relation of ammonia concentration with BALf epithelial cell percentage. It is described in humans that the reaction of water soluble ammonia with moisture on respiratory epithelia creates a corrosive ammonium hydroxide solution (Brautbar, 1998). This could damage respiratory epithelial cells, disrupting the epithelial barrier, resulting in increased susceptibility to infectious pathogenic and opportunistic organisms, predisposing for pulmonary infection (Ackermann et al., 2010; Weitnauer et al., 2015). In contrast to a previous study (Phillips et al., 2010), pulmonary inflammation induced by ammonia exposure, characterised by presence of neutrophils in broncho-alveolar lavage fluid, was not found in our study. Yet, ammonia concentrations in that study were excessively high (15–45 ppm) and thus not comparable to the low concentrations typically present in calf housings, hindering direct comparison of these studies. Future research is needed to unravel the effects of chronic, low concentration ammonia exposure on pulmonary immunity in calves.

Fourth, the effects of air velocity. In practice, draught exposure is considered a very important risk factor for respiratory disease, and the most frequently used cut-off values are maximum velocities of 0.3 m/s (Buczinski et al., 2018) or 0.5 m/s (Lundborg et al., 2005), determined by punctual measurements. However, the peer-reviewed published evidence for these suggested cut-offs is not extensive. Only one study showed an association of air velocity (> 0.5 m/s) with increased respiratory sounds on lung auscultation (Lundborg et al., 2005). Lung auscultation is highly variable between observers (Pardon et al., 2019) and a less accurate diagnostic tool for pneumonia diagnosis compared to thoracic ultrasonography (Buczinski et al., 2014). In contrast to a recent study evaluating the 0.3 m/s cut-off for its association with lung ultrasonographic findings (Buczinski et al., 2018), we did find an association with lung consolidation, albeit at a slightly higher cut-off (> 0.8 m/s). Since air velocity can be highly variable during a given day, mostly depending on outdoor weather conditions in naturally ventilated stables, the association of a one-time punctual measurement was noteworthy. Lung consolidation as detected by ultrasound on a single occasion can either represent acute of chronic pneumonia. It is inherent to a cross-sectional study design, that no causal, or time related inference can be drawn on the association between air velocities > 0.8 m/s and lung consolidation. Furthermore, the effects of air velocity on respiratory disease and thermal comfort of calves are often considered in combination with temperature, as high air velocity has a chilling effect, increasing cold stress by enhancing perception of low temperatures (Da Silva, 2012). Our findings showed no associations of air velocity with temperature and as discussed above the effects of low temperatures itself could also not be demonstrated. A possible pathogenesis could be that draught exposure first induced airway inflammation, which can further develop into pneumonia. We did however not find any association between air velocities above 0.8 m/s and increase in neutrophils of other cell types in BALf. Air velocity was only negatively associated with TNCC, which is the least useful parameter to study airway inflammation, since its concentration is influenced by several other factors such as returned broncho-alveolar lavage volume and presence of blood staining in the BALf sample (van Leenen et al., 2019).

In recent years bacterial air load was introduced as a useful parameter to estimate stable climate and monitor improvements by changes in ventilation type or system (Lago et al., 2006). However, no links could be found for any of the studied outcomes with bacterial air load, resembling a recent study (Buczinski et al., 2018). In our study mean bacterial air load was higher than in the original study showing an association between bacterial air load and clinical signs (on average 247.002 cfu/m^3 vs. 112.280 cfu/m^3) (Lago et al., 2006). The larger stocking density and animal mass in our study, with older calves having a larger body surface, could explain these findings since the major source of bacteria in a barn are the calves themselves (Hill et al., 2011). Considering the isolation of respiratory bacterial pathogens from BALf, it is known that opportunistic bacteria are present in the upper respiratory tract of healthy calves (Griffin et al., 2010). These potential pathogens colonize the airways, and this can proceed to infection when predisposing factors hamper innate immunity of the respiratory tract. However, we could not identify any of the climate parameters which would result in higher isolation rates of these opportunistic respiratory pathogens. Besides disease transmission following exposure to airborne bacteria another possibility should be considered in this context. As stated by others (Lago et al., 2006), a solid barrier between individual calf housings preventing nose-nose contact is associated with decreased prevalence of BRD, as this reduces direct pathogen transmission between calves. Our results could be biased since calves in our study were able to have nose-nose contact as they were group-housed.

Finally, despite its use as an indicator to estimate ventilation rates and efficiency in other species such as pigs, the use of CO_2 as an air quality parameter in calf barns seems limited. Although measured CO_2 concentrations in calf barns reach higher maximum values when compared to pig housings (Van Ransbeeck et al., 2013), no associations with health parameters were found. Moreover, in pig barns NH_3 and CO_2 concentrations are highly positively correlated (Van Ransbeeck et al., 2013) and this correlation was not found in our study. These differences might be attributable to variability in air composition between pig and calf barns, possibly due to different ventilation systems used (mechanical ventilation in pig buildings versus predominantly natural ventilation in calf barns) and differences in floor type and bedding (slatted floors versus straw bedding). Interactions of CO_2 and other parameters such as temperature and draught could not be evaluated in our study due to insufficient power.

Our study was subject to some limitations. Since the majority of farms used a natural ventilation system specific microclimates could exist on different places in the barn. Therefore, results could not always be extrapolated to the barn as a whole or other sites within the barn. For financial reasons viral pathogens were not included and therefore their effects could not be excluded in this study. Other air quality parameters, for instance dust or endotoxins, and viral pathogens should be considered in future studies to further elucidate the complexity of BRD in calves. Finally, measurements in our study were conducted on a single occasion. Despite the fact that farmers were asked not to change normal farm routine or purposely perform additional cleaning activities before our visit, it cannot be excluded that some of these single measurements were not entirely representative of everyday climate conditions. To provide better insights in the climatic changes associated with BRD in calves future studies should consider a longitudinal study design.

5. Conclusion

Of all barn air quality parameters investigated, only temperature, ammonia concentrations above 4 ppm in 24-h measurements and

presence of an air velocity > 0.8 m/s were associated with lung consolidation in group-housed calves. This is supported by the identification of climate clusters, suggesting that draught and warm, dry and increased ammonia climate clusters differ from under-ventilated and presumably normal clusters. This information might aid towards a more evidence based evaluation of barn climate, including continuous measurements, resulting in tailor-made solutions for specific air quality problems.

Funding

This work was done at the large animal clinic of Ghent University with a special research fund (BOF) of Ghent University, granted to K. van Leenen (01D25016). Bacterial species identification was done by MALDI-TOF MS financed by the Research Foundation Flanders (FWO-Vlaanderen) as Hercules project AUGE/15/05 (G0H2516N).

Acknowledgements

The authors would like to thank the cooperating farmers and veterinary practices for their much appreciated help with this project.

References

Ackermann, M.R., Derscheid, R., Roth, J.A., 2010. Innate immunology of bovine respiratory disease. Vet. Clin. North Am. Food Anim. Pract. 26, 215–228. https://doi.org/10.1016/j.cvfa.2010.03.001.

Ames, T.R., 1997. Dairy calf pneumonia. The disease and its impact. Vet. Clin. North Am. Food Anim. Pract. 13, 379–391. https://doi.org/10.1016/S0749-0720(15)30303-0.

Bach, A., 2011. Associations between several aspects of heifer development and dairy cow survivability to second lactation. J. Dairy Sci. 94, 1052–1057. https://doi.org/10.3168/jds.2010-3633.

Baptiste, K.E., Kyvsgaard, N.C., 2017. Do antimicrobial mass medications work? A systematic review and meta-analysis of randomised clinical trials investigating antimicrobial prophylaxis or metaphylaxis against naturally occurring bovine respiratory disease. Pathog. Dis. 75, 1–12. https://doi.org/10.1093/femspd/ftx083.

Beeler-Marfisi, J., Clark, M.E., Wen, X., Sears, W., Huber, L., Ackerley, C., Viel, L., Bienzle, D., 2010. Experimental induction of recurrent airway obstruction with inhaled fungal spores, lipopolysaccharide, and silica microspheres in horses. Am. J. Vet. Res. 71, 682–689. https://doi.org/10.2460/ajvr.71.6.682.

Brautbar, N., 1998. Ammonia exposure: a common cause for sinusitis. A case report and review of the literature. Toxicol. Ind. Heal. 14, 891–895.

Buczinski, S., Forté, G., Francoz, D., Bélanger, A., 2014. Comparison of thoracic auscultation, clinical score, and ultrasonography as indicators of bovine respiratory disease in preweaned dairy calves. J. Vet. Intern. Med. 28, 234–242. https://doi.org/10.1111/jvim.12251.

Buczinski, S.L., Ollivett, T., Dendukuri, N., 2015. Bayesian estimation of the accuracy of the calf respiratory scoring chart and ultrasonography for the diagnosis of bovine respiratory disease in pre-weaned dairy calves. Prev. Vet. Med. 119, 227–231. https://doi.org/10.1016/j.prevetmed.2015.02.018.

Buczinski, S., Borris, M.E., Dubuc, J., 2018. Herd-level prevalence of the ultrasonographic lung lesions associated with bovine respiratory disease and related environmental risk factors. J. Dairy Sci. 101, 2423–2432. https://doi.org/10.3168/jds.2017-13459.

CIGR, 1984. Report of Working Group on Climatization of Animal Houses. Scottisch Farm Building Investigation Unit.

Cramer, M.C., Ollivett, T.L., 2019. Growth of preweaned, group-housed dairy calves diagnosed with respiratory disease using clinical respiratory scoring and thoracic ultrasound—a cohort study. J. Dairy Sci. 102, 4322–4331. https://doi.org/10.3168/jds.2018-15420.

Da Silva, R.G., 2012. Weather and Climate and Animal Production. in: Guide to Agricultural Meteorological Practices. World Meteorological Organization, Geneva, Switzerland, pp. 1–21.

DIN 18910, 2017. Thermal Insulation for Closed Livestock Buildings - Thermal Insulation and Ventilation - Principles for Planning and Design for Closed Ventilated Livestock Buildings. German Institute for Standardisation, VDI-Guideline, Berlin, Germany.

Dohoo, I., Martin, W., Stryhn, H., 2009. Veterinary Epidemiologic Research, second ed. VER Inc, Charlottetown.

Dubrovsky, S.A., Van Eenennaam, A.L., Karle, B.M., Rossitto, P.V., Lehenbauer, T.W., Aly, S.S., 2019. Bovine respiratory disease (BRD) cause-specific and overall mortality in preweaned calves on California dairies: the BRD 10K study. J. Dairy Sci. 102, 7320–7328. https://doi.org/10.3168/jds.2018-15463.

Dunn, T.R., Duffield, T.F., Kelton, D.F., Leslie, K.E., Ollivett, T.L., Renaud, D.L., LeBlanc, S.J., 2018. The effect of lung consolidation, as determined by ultrasonography, on first-lactation milk production in Holstein dairy calves. J. Dairy Sci. 101, 5404–5410. https://doi.org/10.3168/jds.2017-13870.

Egberts, V., van Schaik, G., Brunekreef, B., Hoek, G., 2019. Short-term effects of air pollution and temperature on cattle mortality in the Netherlands. Prev. Vet. Med. 168, 1–8. https://doi.org/10.1016/j.prevetmed.2019.03.021.

Ellis, J.A., 2001. The immunology of the bovine respiratory disease complex. Vet. Clin. North Am. Food Anim. Pract. 17, 535–550. https://doi.org/10.1016/S0749-0720(15)30005-0.

European Medicines Agency and European Food Safety Authority, 2017. EMA and EFSA joint scientific opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety. EFSA J 15, 1–245. https://doi.org/10.2903/j.efsa.2017.4666.

Griffin, D., 1997. Economic impact associated with respiratory disease in beef cattle. Vet. Clin. North Am. Food Anim. Pract. 13, 367–377. https://doi.org/10.1016/S0749-0720(15)30302-9.

Griffin, D., Chengappa, M.M., Kuszak, J., McVey, D.S., 2010. Bacterial pathogens of the bovine respiratory disease complex. Vet. Clin. North Am. Food Anim. Pract. 26, 381–394. https://doi.org/10.1016/j.cvfa.2010.04.004.

Herbut, P., Angrecka, S., 2014. Ammonia concentrations in a free-stall dairy barn. Ann. Anim. Sci. 14, 153–166. https://doi.org/10.2478/aoas-2013-0065.

Hill, T.M., Bateman, H.G., Aldrich, J.M., Schlotterbeck, R.L., 2011. Comparisons of housing, bedding, and cooling options for dairy calves. J. Dairy Sci. 94, 2138–2146. https://doi.org/10.3168/jds.2010-3841.

Kaufman, J., Linington, M., Osborne, V.R., Wagner-Riddle, C., Wright, T.C., 2015. Short communication: field study of air ammonia concentrations in Ontario dairy calf housing microenvironments. Can. J. Anim. Sci. 95, 539–542. https://doi.org/10.4141/cjas-2015-013.

Lago, A., McGuirk, S.M., Bennett, T.B., Cook, N.B., Nordlund, K.V., 2006. Calf respiratory disease and pen microenvironments in naturally ventilated calf barns in winter. J. Dairy Sci. 89, 4014–4025. https://doi.org/10.3168/jds.S0022-0302(06)72445-6.

Louie, A.P., Rowe, J.D., Love, W.J., Lehenbauer, T.W., Aly, S.S., 2018. Effect of the environment on the risk of respiratory disease in preweaning dairy calves during summer months. J. Dairy Sci. 101, 10230–10247. https://doi.org/10.3168/jds.2017-13716.

Lundborg, G.K., Svensson, E.C., Oltenacu, P.A., 2005. Herd-level risk factors for infectious diseases in Swedish dairy calves aged 0-90 days. Prev. Vet. Med. 68, 123–143. https://doi.org/10.1016/j.prevetmed.2004.11.014.

Mäkinen, T.M., Juvonen, R., Jokelainen, J., Harju, T.H., Peitso, A., Bloigu, A., Silvennoinen-Kassinen, S., Leinonen, M., Hassi, J., 2009. Cold temperature and low humidity are associated with increased occurrence of respiratory tract infections. Respir. Med. 103, 456–462. https://doi.org/10.1016/j.rmed.2008.09.011.

McGuirk, S.M., Peek, S.F., 2014. Timely diagnosis of dairy calf respiratory disease using a standardized scoring system. Anim. Heal. Res. Rev. 15, 145–147. https://doi.org/10.1017/S1466252314000267.

Meyer, K.C., 2007. Bronchoalveolar lavage as a diagnostic tool. Semin. Respir. Crit. Care Med. 28, 546–560. https://doi.org/10.1055/s-2007-991527.

Mourtzoukou, E.G., Falagas, M.E., 2007. Exposure to cold and respiratory tract infections. Int. J. Tuberc. Lung Dis. 11, 938–943.

Nonnecke, B.J., Foote, M.R., Miller, B.L., Fowler, M., Johnson, T.E., Horst, R.L., 2009. Effects of chronic environmental cold on growth, health, and select metabolic and immunologic responses of preruminant calves. J. Dairy Sci. 92, 6134–6143. https://doi.org/10.3168/jds.2009-2517.

Ollivett, T.L., Buczinski, S., 2016. On-farm use of ultrasonography for bovine respiratory disease. Vet. Clin. North Am. - Food Anim. Pract. 32, 19–35. https://doi.org/10.1016/j.cvfa.2015.09.001.

Pardon, B., 2019. A quick scan lung ultrasound method and flow chart as a decision aid for bronchopneumonia diagnosis. In: Proceedings of the 24th ANEMBE Conference. Sevilla, Spain. pp. 258–260.

Pardon, B., Hostens, M., Duchateau, L., Dewulf, J., De Bleecker, K., Deprez, P., 2013. Impact of respiratory disease, diarrhea, otitis and arthritis on mortality and carcass traits in white veal calves. BMC Vet. Res. 9, 79. https://doi.org/10.1186/1746-6148-9-79.

Pardon, B., Buczinski, S., Deprez, P.R., 2019. Accuracy and inter-rater reliability of lung auscultation by bovine practitioners when compared with ultrasonographic findings. Vet. Rec. 185, 1–4. https://doi.org/10.1136/vr.105238.

Phillips, C.J.C., Pines, M.K., Latter, M., Muller, T., Petherick, J.C., Norman, S.T., Gaughan, J.B., 2010. The physiological and behavioral responses of steers to gaseous ammonia in simulated long-distance transport by ship. J. Anim. Sci. 88, 3579–3589. https://doi.org/10.2527/jas.2010-3089.

Rawson, R.E., Dziuk, H.E., Good, A.L., Anderson, J.F., Bates, D.W., Ruth, G.R., Serfass, R.C., 1989. Health and metabolic responses of young calves housed at -30 degrees C to -8 degrees C. Can. J. Vet. Res. 53, 268–274.

Schnyder, P., Schönecker, L., Schüpbach-Regula, G., Meylan, M., 2019. Effects of management practices, animal transport and barn climate on animal health and antimicrobial use in Swiss veal calf operations. Prev. Vet. Med. 167, 146–157. https://doi.org/10.1016/J.PREVETMED.2019.03.007.

Seedorf, J., Hartung, J., 1999. Survey of ammonia concentrations in livestock buildings. J. Agric. Sci. 133, 433–437. https://doi.org/10.1017/S0021859699007170.

Seedorf, J., Hartung, J., Schröder, M., Linkert, K.H., Pedersen, S., Takai, H., Johnsen, J.O., Metz, J.H.M., Groot Koerkamp, P.W.G., Uenk, G.H., Phillips, V.R., Holden, M.R., Sneath, R.W., Short, J.L., White, R.P., Wathes, C.M., 1998. Temperature and moisture conditions in livestock buildings in Northern Europe. J. Agric. Eng. Res. 70, 49–57. https://doi.org/10.1006/jaer.1997.0284.

Siegel, S.J., Weiser, J.N., 2015. Mechanisms of bacterial colonization of the respiratory tract. Annu. Rev. Microbiol. 69, 425–444. https://doi.org/10.1146/annurev-micro-091014-10420.

Teixeira, A.G.V., McArt, J.A.A., Bicalho, R.C., 2017. Thoracic ultrasound assessment of lung consolidation at weaning in Holstein dairy heifers: reproductive performance and survival. J. Dairy Sci. 100, 2985–2991. https://doi.org/10.3168/jds.2016-12016.

Van Driessche, L., Valgaeren, B.R., Gille, L., Boyen, F., Ducatelle, R., Haesebrouck, F.,

Deprez, P., Pardon, B., 2017. A deep nasopharyngeal swab versus nonendoscopic bronchoalveolar lavage for isolation of bacterial pathogens from preweaned calves with respiratory disease. J. Vet. Intern. Med. 31, 946–953. https://doi.org/10.1111/jvim.14668.

van Leenen, K., Van Driessche, L., De Cremer, L., Gille, L., Masmeijer, C., Boyen, F., 2019. Factors associated with lung cytology as obtained by non-endoscopic broncho- alveolar lavage in group-housed calves. BMC Vet. Res. 15, 167.

van Leenen, K., Van Driessche, L., De Cremer, L., Masmeijer, C., Boyen, F., Deprez, P., Pardon, B., 2020. Comparison of bronchoalveolar lavage fluid bacteriology and cytology in calves classified based on combined clinical scoring and lung ultrasonography. Prev. Vet. Med. 176, 104901. https://doi.org/10.1016/j.prevetmed.2020.104901.

Van Ransbeeck, N., Van Langenhove, H., Demeyer, P., 2013. Indoor concentrations and missions factors of particulate matter, ammonia and greenhouse gases for pig fattening facilities. Biosyst. Eng. 116, 518–528. https://doi.org/10.1016/j.biosystemseng.2013.10.010.

Virtala, A.M.K., Gröhn, Y.T., Mechor, G.D., Erb, H.N., 1999. The effect of maternally derived immunoglobulin G on the risk of respiratory disease in heifers during the first 3 months of life. Prev. Vet. Med. 39, 25–37. https://doi.org/10.1016/S0167-5877(98)00140-8.

Weitnauer, M., Mijošek, V., Dalpke, A., 2015. Control of local immunity by airway epithelial cells. Mucosal Immunol. 9, 287–298. https://doi.org/10.1038/mi.2015.126.

WHO, 2001. WHO Global Strategy for Containment of Antimicrobial Strategy for Containment of Antimicrobial Resistance 105. World Health WHO/CDS/CS, pp. 99 https://doi.org/WHO/CDS/CSR/DRS/2001.2.

Windeyer, M.C., Leslie, K.E., Godden, S.M., Hodgins, D.C., Lissemore, K.D., LeBlanc, S.J., 2012. The effects of viral vaccination of dairy heifer calves on the incidence of respiratory disease, mortality, and growth. J. Dairy Sci. 95, 6731–6739. https://doi.org/10.3168/jds.2012-5828.

Windeyer, M.C., Leslie, K.E., Godden, S.M., Hodgins, D.C., Lissemore, K.D., LeBlanc, S.J., 2014. Factors associated with morbidity, mortality, and growth of dairy heifer calves up to 3 months of age. Prev. Vet. Med. 113, 231–240. https://doi.org/10.1016/j.prevetmed.2013.10.019.