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Master's thesis

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An observational study of the effect of a *Mycoplasma bovis* outbreak on the reproduction performance at herd level

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Tobias Volhøj, Tårs d. 15-01-2015

Tobias V

Foreword

In the fall of 2014, 3 master students (Lene Jensen, Bolette Troldborg Rafn and I) were studying different aspects of *M. bovis*. To begin with, we created a questionnaire where Lene was the main responsible. I have used the answers to a few of these questions, which can be seen below in this thesis.

The thesis is written as an article, meaning that there is no background chapter and the intro chapter is a bit larger instead.

It has caught my attention that farmers and veterinarians have focused heavily on *M. bovis* during the last couple of years. This and my personal interest in breeding and reproduction is the reason why I chose this subject.

An interesting side effect of the outbreaks starting in 2010 is quite well described by a conversation I had with a veterinarian. The practitioner told me, that she believes that after there has become such a big focus on *Mycoplasma* in Denmark they suddenly found a few cases of *M. bovis* mastitis. Before 2010 where *M. bovis* started getting a lot of focus, they would probably just have seen it as a mastitis non-responsive to antibiotics and sent the cow to the slaughterhouse. This is one of many things the outbreaks have had an impact on. Another thing could be the increasing interest of biosecurity including trading with animals.

The project has been made possible by economical and technical support of The Knowledge Center of Agriculture, Cattle, Agrotech and KU Sund.

I would like to thank my supervisors, Liza Rosenbaum Nielsen, Matt Denwood and Kaspar Krogh, and special consultant Marie Louise Ancker for all the great support and inputs.

I would also like to thank everyone else who have bothered to help me through this thesis, amongst them especially my family.

Resumé

Det er kendt at *Mycoplasma bovis* (*M. bovis*) kan forårsage forskellige kliniske tegn hos kvæg, såsom mastitis, arthritis og otitis media. Det er dog en begrænset mængde artikler der er udgivet om effekten af *M. bovis* på reproduktionsresultaterne i en malkekvægs besætning.

Formålet med opgaven var at estimere effekten af et *M. bovis* udbrud på reproduktionsresultaterne i malkekvægsbesætninger. Effekten blev evalueret på baggrund af parametrene: drægtighed ved første inseminering (CRFS), intervallet fra kælvning til første inseminering (CFS) og drægtighedslængde (GL). Disse parametre er meget brugte inden for reproduktions arbejdet og er tidligere blevet brugt til at evaluere reproduktionsresultaterne i besætninger, der har været udsat for en smitsom sygdom.

Denne undersøgelse startede med et spørgeskema for at finde malkekvægs besætninger, som havde oplevet et udbrud af *M. bovis*, og for blandt andet at få start og slutdatoer af *M. bovis* udbruddene i de forskellige besætninger. Ud af de 324 besætninger som svarede på spørgeskemaet, havde 123 besætninger oplevet et udbrud. Nogle af disse besætninger blev ekskluderet på baggrund af forskellige kriterier såsom kort varighed af udbruddet eller manglende reproduktions data af tilstrækkelig kvalitet. I den endelige model blev der regnet på 60 besætninger. Reproduktionsresultaterne i en periode omkring start datoen af udbruddet blev sammenlignet med reproduktionsresultaterne i lignende periode året før, for at bruge besætningerne som sin egen reference. Til at analysere parametrene blev der brugt forskellige logistiske og lineære regressions modeller med tilfældige effekter.

Resultaterne af denne undersøgelse viste ikke nogen signifikant ændring (p-værdi <0,05) i CRFS eller GL under udbrudsperioden i forhold til året før. CFS var øget under udbrudsperioden i forhold til året før. Odds ratio for at have et CFS længere end 71 dage under udbruddet sammenlignet med året før var 1,11 (p-værdi: 0,02). CFS blev også analyseret som et kontinuert udfald og det viste en gennemsnitlig stigning i CFS for køer, som kælvede omkring start datoen på udbruddet, med 2,6 dage (p-værdi: <0,01) i forhold til året før.

Det vides ikke om ændringerne i reproduktionsresultaterne var en direkte følge af *M. bovis*, eller om *M. bovis* havde påvirket køernes velbefindende på anden vis. Det vides heller ikke om det var et resultat af landmændenes påvirkning af udbruddene.

Opgaven er struktureret som et manuskript-udkast, hvorfor abstract først kommer efter indholdsfortegnelsen.

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Abstract

Mycoplasma bovis (*M. bovis*) is known to cause different clinical signs in cattle such as mastitis, arthritis and otitis media. However, there is limited research published on the effect of *M. bovis* on the reproduction performance.

The objective of this study was to estimate the effect of a *M. bovis* outbreak on the reproduction results in dairy herds. The effect was evaluated on the following parameters: conception risk at first service (CRFS), calving to first service (CFS) interval, and gestation length (GL).
These parameters are well known and have previously been used to evaluate the reproduction results of a herd exposed to a contagious disease.

This study started with a questionnaire to identify dairy herds that had experienced a *M. bovis* outbreak, and to get start and end dates of *M. bovis* outbreaks in different herds. Out of a total of 324 responding dairy herds, some of the 123 herds that had experienced an outbreak were excluded based on different criteria, such as short duration of the outbreak or lack of sufficient reproduction data quality. A total of 60 herds were included in the final models. The reproduction results of a period around the start date of the outbreak were compared to a similar period one year before to ensure the herds worked as their own reference. The parameters were analyzed using different mixed logistic and linear regression models.

The results of this study did not demonstrate any significant change in the CRFS or GL during the outbreak period compared to the year before. The CFS interval increased during the outbreak period compared to the year before. The odds ratio of having a CFS longer than 71 days during the outbreak period compared to the year before was 1.11 (p-value: 0.02). CFS was also analyzed as a continuous outcome and it showed an avarage increased CFS interval for cows calving close to or during the outbreak of 2.6 days (p-value <0.01) compared to the year before.

We do not know if the changes in reproductive performance were directly due to *M. bovis* or if *M. bovis* had affected the cows wellbeing in other ways. Neither do we know if it was a result of the farmers’ response to the outbreaks.

Keywords: *Mycoplasma bovis*, outbreak, reproduction

1. Introduction

In the years 2010 to 2014, there have been some serious *Mycoplasma bovis (M. bovis)* outbreaks in Denmark with several animals euthanized or culled. As a result of these outbreaks, a great focus on the disease arose among the farmers and the whole cattle industry, which has brought along a lot of research on the disease.

This thesis is a part of that research and it will focus on how the outbreaks have influenced the reproduction in dairy herds that has experienced *M. bovis* outbreaks.

The farmers' organization has been in charge of most of the research and they have been in contact with many of the farmers experiencing outbreaks. Apart from well-known clinical signs such as mastitis, pneumonia, arthritis and otitis media, some of the experiences the farmers reported from the outbreak include (Krogh, 2014):

1. Fewer inseminated cows
2. Inseminated cows not getting pregnant
3. Abortions
4. Calves born with congenital deformations or signs of arthritis

In the literature, there is limited research that reports an effect of *M. bovis* on reproduction at herd level.

In one study, it was found that cows seropositive to *M. bovis* more often had long intervals from calving to pregnancy diagnosis compared to seronegative cows (Uhaa et al., 1990).

Another study found three of 22 aborted fetuses (13.6%) and 16.7% of 12 placentae of aborted fetuses test positive for *M. bovis* by culture and immunofluorescent techniques (Hassan and Dokhan, 2004).

All of this has contributed to my decision of making a more systematic evaluation of the reproduction in the affected herds during a period related to the outbreak in the herd. Since most cows with clinical signs are culled almost immediately it is primarily the effect on subclinicaly infected cows we are investigating in this study.

The objective of this study is to clarify the effects of an *M. bovis* outbreak on herd reproduction parameters.

1.1 *Mycoplasma bovis (M. bovis)*

The prokaryote organisms *Mycoplasmas* are a part of the class *Mollicutes*, which contains all degenerated and wall-less bacteria, like *Ureaplasmas*. *Mycoplasmas have* a very small genome size and are living without a cell membrane, which disables the effect of β-lactams. The sensitivity and specificity of diagnostic methods are unknown but are generally regarded as lower than for other bacteria (Nicholas et al., 2008). The sensitivity and specificity depend on the kit used.
It has been possible to kill the bacteria in vitro with well-known antimicrobials like oxytetracykline, tylosin and enrofloxacin, but it has not proven effective in vivo (Pfutzner and Sachse, 1996).

In June and July 2013, all Danish dairy herds were screened for antibodies against *M. bovis* by testing antibody level in bulk tank milk. The screenings were performed by the Danish dairy farmers' organization (Knowledge Centre for Agriculture, Cattle (VFL)). They found that 21 % of the Danish dairy herds had a positive ELISA reaction (>37 ODC %) on the bulk tank milk (Anonymous, b, 2013).

Other countries around the world have reported a large variation in the prevalence, e.g. in Flanders, Belgium, they found a herd prevalence of 1.5%, based on PCR (Passchyn et al., 2012). In Seattle/Washington, they found a prevalence of 20 % by culture of bulk tank milk (Fox et al., 2003). When seeing this great variance in results one should take into account that they have used different methods to detect the bacteria. Since the bacteria are shed intermittently and the farmers often withhold milk with visible changes, these prevalences might be underestimating the true prevalence (Maunsell et al., 2011).

M. bovis is primarily causing respiratory disease, both as a primary and a secondary pathogen but it is also observed to cause other clinical manifestations such as mastitis, arthritis, otitis media and reproductive disorders. It is common for all these clinical manifestations of *M. bovis* that the epidemiology and pathophysiology is not fully understood (Nicholas, 2011). *M. bovis* does not always cause clinical disease. It can be found in the upper respiratory tract of clinical healthy animals, which is one of the reasons why it is difficult to monitor and eradicate the bacteria. There have been experiments infusing bacteria from healthy animals into the udder of other healthy animals, which resulted in mastitis. Likewise, it has been shown to cause abortions when infused into the uterus of healthy animals (Pfutzner and Sachse, 1996). Results indicate that latently infected animals probably play a big role in the dissemination of the bacteria (Pfutzner and Sachse, 1996).

The transmission of the bacteria is believed to happen by direct contact between animals, by aerosols over short distances and through contaminated milk or milking equipment. The bacteria are normally found in the nose and the eye of affected animals, which often get in contact with the others animals in the group. Bacteria have been found in the air in a stable with sick animals. The bacteria are assumed to transmit from one udder to another by the milking machine and from infected cows to calves that drink contaminated milk (Maunsell et al., 2011).

It is not fully clarified how well *M. bovis* survives in the environment, but it has been proven to be infective after being frozen in sperm without antibiotics in liquid nitrogen, and to survive for several weeks in milk and on straw (Pfutzner and Sachse, 1996). It has been shown that *M. bovis* can form biofilm, which makes it more resistant to heat and desiccation. It indicates that the bacteria might be capable of surviving for a long time in the environment (McAuliffe et al., 2006).

Generally, *Mycoplasmas* are considered extremely host specific, but *M. bovis* has been shown to pass between goats, sheep and cattle. It is not considered a human pathogen, even though some scientists have reported presence of animal *Mycoplasmas* in immunocompromised humans. Therefore, *M. bovis* should not be excluded as an opportunistic zoonosis (Pitcher and Nicholas, 2005). In 2007 *M. bovis* was reported in pigs, as the only occurring pathogen causing conjunctivitis, nasal discharge and coughing (Spergser et al., 2013).

1.1.1 *M. bovis* and reproduction

The effect of *M. bovis* on reproduction at herd level has not been very well described in the literature, but there is limited evidences that supports that *M. bovis* is an important part of naturally occurring bovine reproductive disease (Maunsell et al., 2011)

However, there are several rapports of the presence of *M. bovis* in aborted fetuses. *M. bovis* has been reported as the only infectious agent isolated from an aborted fetus (Byrne et al., 1999). Watson et al. (2012) investigated aborted fetuses and those that normally would have been declared sterile by a routine investigation were checked for *Mycoplasmas* and *Ureaplasmas*. They found *M. bovis* in two of these aborted fetuses. They suggested that because a routine investigation of aborted fetuses does not include a check for *M. bovis*, the occurrence of *M. bovis* abortions might be underestimated. They concluded that *M. bovis* is seen only as a cause of sporadic abortions compared the infectious agents like *Neospora caninum*.

In a study by Uhaa et al. (1990), blood samples from 572 cows scattered on four different farms were sampled. In total 94 (38%) were tested positive for antibodies directed against *M. bovis* by ELISA.

It was shown that the group of seropositive cows on average was 3 months older at first calving, had a 79 days longer interval from calving to pregnancy diagnosis and an average of 11 days longer intervals from calving to last service compared to the seronegative group. The study did not consider other factors.

In another study, no correlation was found between having cows or heifers in a herd that were seropositive to *M. bovis* and the performance of the herd according to abortions and insemination index. Insemination index was defined as the number of inseminations pr. pregnancy (Raaperi et al., 2012).

1.2 Reproduction

When the reproduction of a herd is evaluated and benchmarked to other herds there are many factors that one have to consider the impact, of since the reproductive parameters we use in our herds are greatly influenced by management, environment, genetics and many other factors (Löf, 2012).

1.2.1 Parameters

Conception risk at first service (CRFS) is an often used reproductive parameter (VanLeeuwen et al., 2010); (Fourichon et al., 2000); (Aungier et al., 2014). It is calculated by dividing the number of pregnancies (after first service) with the number of first services.

CRFS is affected by many factors, such as the number of previous ovulations, the staff's ability to detect heat, body condition score and the health status of the cow (Aungier et al., 2014); (Noakes et al., 2001). One disadvantage of the parameter is that you need to have a pregnancy confirmation; otherwise, you will have to use historic data where you use calving date and see if it fits the day of the insemination. When doing this you will miss the cows that have aborted and been sold or slaughtered afterwards without getting rebred. All cows that the farmer wants to breed are included in this parameter (Löf, 2012).

There are not many first services every week, but it tells something about the status of the cow after calving, which is a very critical period (Aungier et al., 2014). It is usually the time where we have the highest conception rate because later on the infertile cows will be a larger proportion of the cows that are inseminated.

Calving to first service (CFS) is used worldwide as well (Fourichon et al., 2000; Löf, 2012) and has among others, been used to measure the effect of bovine respiratory syncytial virus (BRSV) and bovine corona virus (BCV) on reproductive performance (Ohlson et al., 2010).

CFS is the interval in days between the day the cow calves and the first service. It is affected by the farmer and his choice of voluntary waiting period (VWP) before he starts to inseminate the cows. CFS is also affected by cows that does not return to cycle and by the farmer's skills of detecting heat (Noakes et al., 2001).

Cows are more receptive to disease around the time of calving and returning to cycle is affected by the wellbeing of the cow (Aungier et al., 2014). It is interesting to evaluate CFS since it is related to the observation closest related to calving. Newly calved cows have been shown to be one of the most affected groups when outbreaks of BRSV occurs (Elvander, 1996).

Gestation length (GL) has been investigated in many studies especially in relation to calving difficulty, stillbirth and abortion. Gestation length has for example been used to describe the effect of infection with bluetongue virus (Hansen et al., 2004; Nusinovici et al., 2012).

GL is calculated as the number of days from the pregnancy giving insemination until the day of birth. The gestation is divided in an embryonic period (day 0 – 43) and a foetus period (day 43 – 260). The calf is considered viable from day 260, but it depends on the maturation of the organs (Ancker and Agerholm, 2010).

The mean gestation length of Danish first parity Holsteins is 278.5 days (Hansen et al., 2004). By looking at the end of the gestation, it is only cows that the farmer has decided to keep that are investigated.

2. Materials and methods

2.1 Overview of the Danish dairy cattle population

Data were collected from herds delivering milk during the years 2009 – 2014. In 2012 there were about 587,000 dairy cows in Denmark distributed in approximately 3,886 herds. In total approximately, 71% of the dairy cows are Danish Holstein, 13% are Jersey and 7% are Danish Red. The rest are less common breeds and cross breeds (Pedersen, 2014). The cows are typically fed total mixed rations and are housed in loose housing barn systems all year arround. The average milk production of a Danish dairy cow was 9,138 kg milk per year in 2012/2013 (Anonymous, a, 2014). The reproduction results in Denmark, based on cows that calved in 2005, showed the mean CFS interval to be 94.7 days, CRFS was 41.3% and the reproduction efficiency was 0.15 (Ancker, 2008).

2.2 Sampling

A questionnaire was constructed as a part of a master thesis for investigation of factors associated with outbreaks of *M. bovis* (Jensen, 2015). I was a part of the construction and had three questions added (b, c and d). The answers of the following questions were used in this thesis:

a) When did the outbreak of *Mycoplasma bovis* according to your best belief occur? You can indicate an interval. (In Danish: Hvornår har udbruddet med Mycoplasma bovis efter din bedste overbevisning stået på? Du må gerne angive et interval.)
   1. approximate start date of clinical cases:
   2. approximate end date of clinical cases:

b) Have any changes been made to the reproduction management from a year before to a year after the outbreak of *Mycoplasma* bovis? (E.g. flushings, change of artificial inseminator or new person in charge of the reproduction). (In Danish: Er der lavet om i reproduktionen fra et år før til et år efter udbruddet af *Mycoplasma bovis*? (skylninger, ejer-inseminør, ny ansvarlig for reproduktionsarbejdet))
   1. No
   2. Yes

c) Have you stopped/started using Heat time? (In Danish: Er der stoppet/påbegyndt brug af Heat time?)
   1. No
   2. Yes, approximate date:

d) Have you stopped/started using a bull for the cows? (In Danish: Er der stoppet/påbegyndt brug af egen tyr?)
   1. No
   2. Yes, approximate date:

The herd had to meet at least one of the following inclusion criteria for participation in the questionnaire study.

1. The herd had reported an outbreak suspected to be *M. bovis* to the Knowledge Center for Agriculture, Cattle (VFL).
2. The herd had participated in previous projects about *M. bovis*, i.e. projects managed by VFL.
3. The herd had had one or more positive *M. bovis* tests (PCR < Ct 40 or Elisa > 55 ODC %) on bulk tank milk samples. The samples could origin from one of three national screening rounds, self-requested samples or samples tested before an animal shows (a requirement for participation in Danish animal shows, in 2014).

A total of 504 herds met the criteria. The herds that met the criteria were phoned and given the opportunity to receive an e-mail with a link to the questionnaire or to answer the questionnaire over the phone, with the interviewer when it was most convenient for the farmer. The questionnaire was tested on some farmers that did not meet the criteria and adjusted. The answers have not been validated by visiting the herds and checked if their answers were consistent with what is seen on the farm (Jensen, 2015). Some herds were excluded afterwards based on criteria for participation in this study. The final dataset for analysis included 60 dairy herds. The exclusion criteria and processes are illustrated in Figure 1.

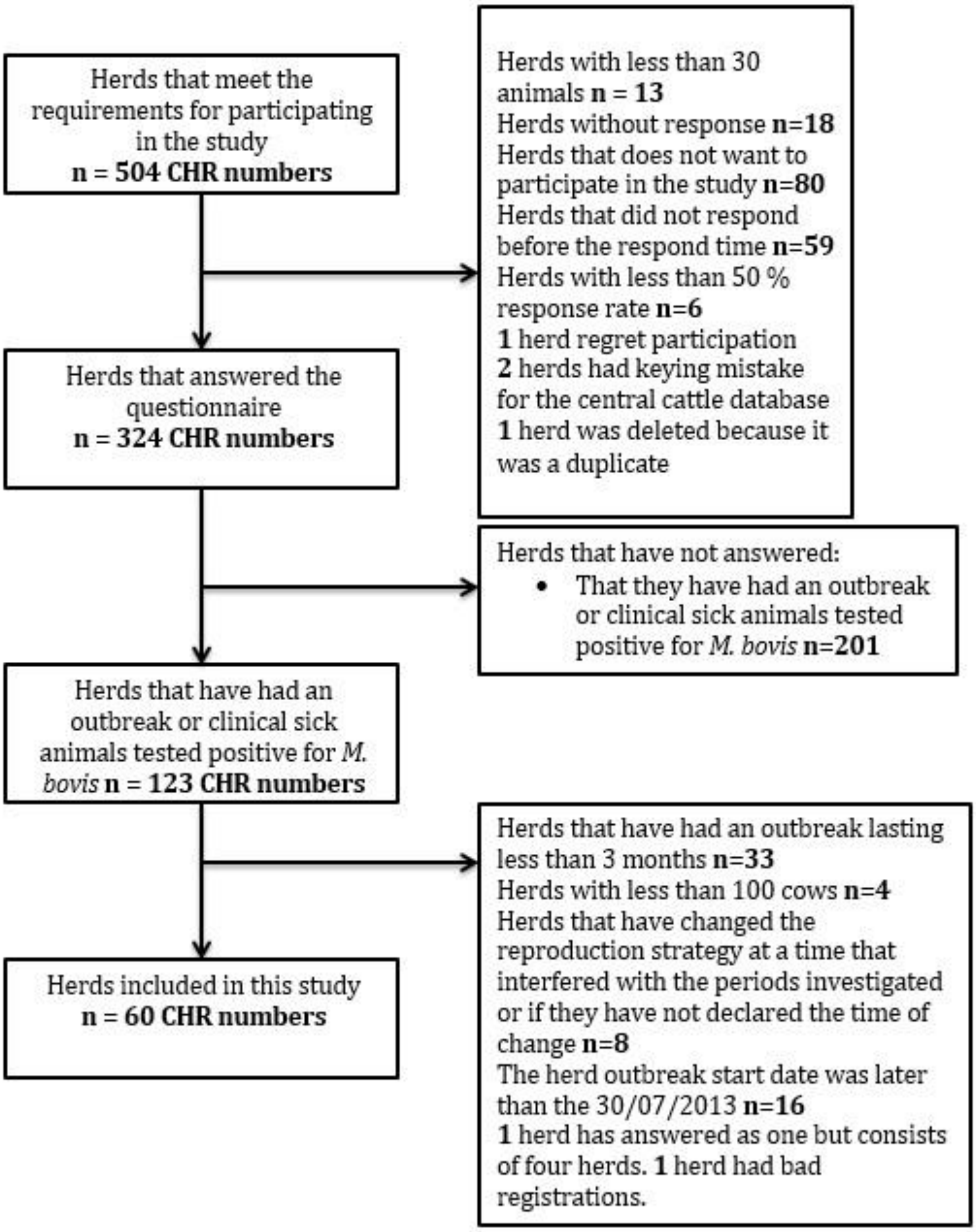

Figure 1
Flowchart showing the exclusion of herds that met the original criteria.

2.3 Observation periods

The reproduction results of a herd, during the outbreak, were compared to the reproduction results of a period one year before with similar duration as the outbreak period. By doing this and by excluding herds that reported having changed reproduction procedures, it was assumed that the management of the reproduction and the environment of the cows were almost the same. Hence we could compare the two periods without considering too many other herd level factors.

The introduction of the pathogen, and thereby the possible effect on the animals may not be evident at first. Since it might take some time before the farmer realizes there is an outbreak of *M. bovis* in the herd, the cow reproduction events during the two months before the outbreak start date, reported by the farmer were included. This was also to ensure a sufficient number of calving events for each herd. Furthermore, from the time of calving until the first reproduction events occur usually takes around 15 days, and that is a silent heat, for a well-nourished cow with no complications at calving (Crowe, 2008).

The observation period of interest depended on the reproduction parameter. Since the cows were included in the period based on the date of calving, the observation period was chosen to be from 2 months before the start date of the outbreak and up until 3 months after the start date of the outbreak for the parameters CRFS and CFS, giving equally long observations periods for all the included herds.

The observation period for the parameter GL was set to 15 days before the outbreak start date and up until 3 months after. For this parameter, the analysis was aimed at testing whether there was any effect on the gestation length of the cows when they were affected by the pathogen towards the end of their gestation. The assumption was that cows that calved in this observation period had been exposed to the bacteria at some point in the end of their gestation.

2.4 Variables

The data used for analysis originated from the Danish Cattle Database (DCD). The data received included information on dates of reproductive events, calving information and information about the breed, date of birth, calving date and calving number. Only artificial inseminations were included in this study as services.

The dataset the analysis were based on, consisted of the variables “herd number”, “animal id”, “calving date”, “outbreak start date”, “outbreak end date”, “first insemination date”, “next calf born”, “previous insemination date”, “number of inseminations”, “number of positive pregnancy diagnosis”, “number of negative pregnancy diagnosis”, “period”, “calving to first insemination interval” (CFS), “gestation length” (GL), “conception risk at first service” (CRFS), “parity” and “calving difficulty”.

Period depended on the date of calving and the outbreak start date of the herd as described above. CFS below 30 days was registered as missing (~2.3% of all observations) because it was considered likely to be an erroneous registration (Dohoo et al., 2001). The distribution of the remaining CFS-observations can be seen in Figure 2.

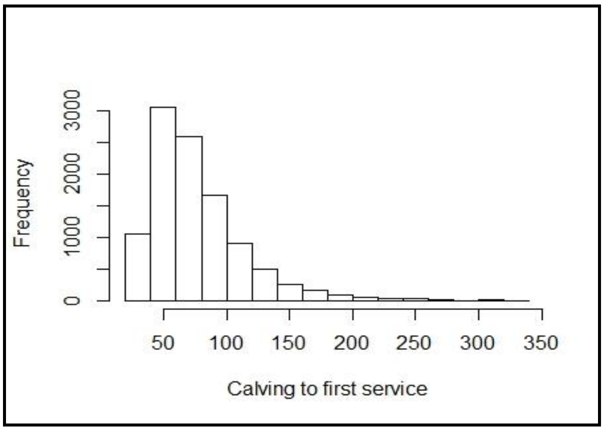

Figure 2
Histogram displaying the distribution of calving to first service intervals (CFS) after removing CFS below 30 days of the cows included in the final model.

Gestation length was calculated from the date of the last insemination to the calving date. It was set to missing if it was registered to be below 260 days (~2.5% of all observations) and over 300 days (~3.1% of all observations) to avoid erroneous registrations. The distribution of GL after the removal can be seen in Figure 3.

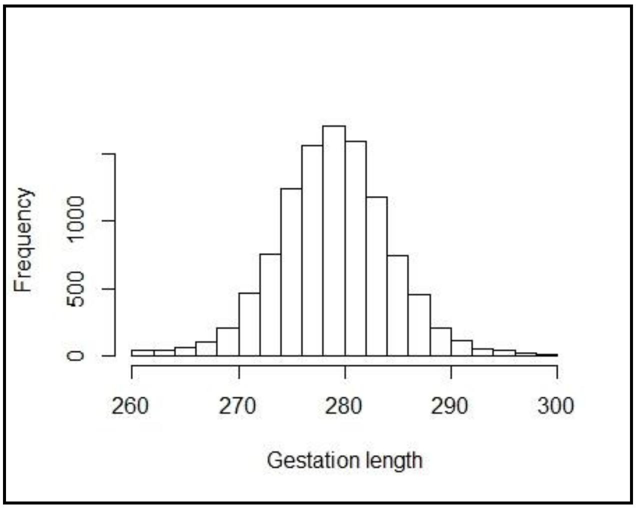

Figure 3
Histogram displaying the distribution of gestation length (GL) after removing observations with GL below 260 days and over 300 days of the cows included in the final model.

The CFS was calculated as the difference in days between date of calving and date of first service. A cow was considered to have conceived at first service, if the cow had a gestation length between 260 and 300 days and only one insemination record between the two calvings. If the registration of the following gestation was missing, the cow was considered to have conceived at first service, if only one insemination and one positive pregnancy diagnosis were recorded. Parity of three and higher were grouped in parity 3+, as well was the variable calving difficulty of three and higher because there were few registrations above three in both groups. Calving difficulty one was described as easy without assistance, two was described as easy with assistance, three was described as difficult without veterinary assistance, four was described as difficult with veterinary assistance and calving difficulty five was a caesarian section. All values that were meaningless were deleted to exclude wrong registrations for example GL or CFS = zero, animals with missing animal id or herd number and duplicates.

2.5 Statistical analysis

All the statistical analyses were performed in R® (R Development Core Team) software (R Development Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2009).

Binomial distributed data such as CRFS were analyzed by logistic regression using the glmer function in R studio (Version 0.98.1062 - © 2009-2013 RStudio, Inc.). The logistic regression model included the effects of period, parity, calving difficulty and the random effect of herd number and animal id. The random effects were included because some animals calved in both periods and because one herd most likely had the same management approaches, stables and health status (beside the *M. bovis outbreak*) in the two periods leading cows within herds to be more alike than cows between herds. Because the random effect of herd was included to account for baseline differences between herds, it was decided not to include the herd level variable breed. Twin pregnancies were not taken into account.

The variables GL and CFS were divided into two levels with a break point at respectively 272 and 71 days. They could then be analyzed by logistic regression as a binomial outcome. To substantiate the break points an evaluation of the effects of using different break points on the model results were analyzed (Figure 4 and Figure 5).

The logistic regression model of GL included the effects of period, parity and random effect of herd number and animal id. The logistic regression model of CFS included the effects of period, parity, calving difficulty and the random effects of herd number and animal id.

CFS was also analyzed with a multivariable mixed linear regression model as a pseudo continuous outcome with gamma distribution, with the same effects as the model analyzing CFS as a binomial outcome.

The models of CRFS and CFS, were tested for interaction between calving difficulty and parity as well as parity and period and period and calving difficulty. If the p-value of the interaction was above 0.05, the interaction term was rejected. The sensitivity of the models to the choice of comparison period was tested by re-running the models using two other reference periods: 1) five months just before and 2) five months after ‘the year before’ period and comparing results between the models. The model of GL was tested for interaction between parity and period. If the p-value of the interaction was above 0.05, the interaction was rejected. The sensitivity of the model to the choice of reference period was tested with two periods of 3.5 months just before and after the year before period.

3. Results

3.1 Descriptive statistics

Descriptive analyses were performed for each parameter and for all the variables included in the final formulas (Tables 1, 2, 3, 4, 5 and 6). There was a difference in the number of animals in the parameters CRFS and CFS, because inseminations earlier than 30 days after calving were registered missing in CFS, but not in CRFS.

Table 1
Descriptive statistics of herd size and outbreak duration in the 60 included dairy herds

Parameter	Min	Median	Mean	Max
Herd size*	**101**	**239**	**260**	**582**
Herd outbreak duration (Months)	**3**	**6**	**8.8**	**36**

* Herd size is the average number of cows recorded in the herd the quarter before the quarter including the start date of the outbreak

Table 2

Descriptive statistics of the fixed effects included in the final model for analyzing the effect of *M. bovis* outbreaks on conception risk at first service (CRFS). Only 59 dairy herds were included, since one herd did not register calving difficulty. The period length was five months.

Parameter	Factor	Year before outbreak period		*M. bovis* outbreak period	
		% that conceived at first insemination	n	% that conceived at first insemination	n
Cows calving in the period			5,321		5,386
Parity	1	0.34	2,127	0.32	2,135
	2	0.31	1,529	0.33	1,603
	3+	0.37	1,665	0.34	1,648
Calving difficulty	Easy without assistance	0.35	4,222	0.33	4,479
	Easy with assistance	0.30	975	0.35	798
	Difficult	0.25	124	0.20	109

Table 3

Descriptive statistics of the continuous variable included in the second model for analyzing the effect of *M. bovis* outbreaks on calving to first service (CFS). Only 59 dairy herds were included, since one herd did not register calving difficulty. The period length was five months.

Parameter	Min*	Median	Mean	Max
Calving to first service (Days) n=10447	30	69	77.5	334
Year before outbreak, n=5175	30	67	77.5	328
***M. bovis* outbreak period, n=5272**	30	69	78	334

* The minimum number of days between calving and first service was constructed by removal of all observations with values below 30 days (~2.3% of the total amount of observations).

Table 4
Descriptive statistics of the categorical variables included in the final model for analyzing the effect of *M. bovis* outbreaks on calving to first service (CFS). Only 59 dairy herds were included, since one herd did not register calving difficulty. The period length was five months.

Parameter	**Factor**	**Year before outbreak period**		***M. bovis* outbreak period**	
		% with CFS interval >71	**n**	**% with CFS interval >71**	**n**
Cows calving in the period			**5,175**		**5,272**
Parity	**1**	**0.44**	**2,073**	**0.46**	**2,083**
	2	**0.44**	**1,480**	**0.45**	**1,571**
	3+	**0.48**	**1,622**	**0.49**	**1,618**
Calving difficulty	**Easy without assistance**	**0.45**	**4,114**	**0.47**	**4,382**
	Easy with assistance	**0.44**	**940**	**0.45**	**782**
	Difficult	**0.50**	**121**	**0.53**	**108**

Table 5
Descriptive statistics of the continuous variable before the breakpoint of 271 days was incorporated in the final model for analyzing the effect of *M. bovis* outbreaks on gestation length (GL). Data of 60 dairy herds for 3.5 months were included in each period.

Parameter	**Min**	**Median**	**Mean**	**Max**
Gestation length (Days) n=7404 Total	**260**	**279**	**279.5**	**300**
Year before outbreak, n=3620	**260**	**280**	**279.5**	**300**
***M. bovis* outbreak period, n=3784**	**260**	**279**	**279.5**	**300**

Table 6
Descriptive statistics of the categorical variable included in the final model for analyzing the effect of *M. bovis* outbreaks on gestation length (GL). Data of 60 dairy herds for 3.5 months were included in each period.

Parameter	Factor	Year before outbreak period		*M. bovis* outbreak period	
		% with gestation length ≤272	n	% with gestation length ≤272	n
Cows calving in the period			3,620		3,784
Parity	1	0.12	1,291	0.13	1,392
	2	0.07	1,112	0.08	1,163
	3+	0.06	1,217	0.06	1,229

3.2 Evaluation of cut-points for dichotomized explanatory variables

The effect of the period was quite constant at the different points around the chosen breakpoints Figure 4 and Figure 5. Biologically it makes sense to say that a gestation length below 272 days is short. In 2004 it was found that the average GL of 168,442 first parity Danish Holstein cows were 278.5 (SD 5.088) (Hansen et al., 2004). The overall proportion of GL ≤ 272 days in this study was 8.9%.

The reproduction results in Denmark, based on cows that calved in 2005 showed that the mean number of days from calving to start insemination was 49 days (Ancker, 2008).

The fact that many farmers use 50 days as the length of the voluntary waiting period and then all cows have one cycle (21+50 days) where they most likely were eligible to service supports the breakpoint of 71 days. A total of 45.9% of the total amounts of CFS intervals were > 71 days.

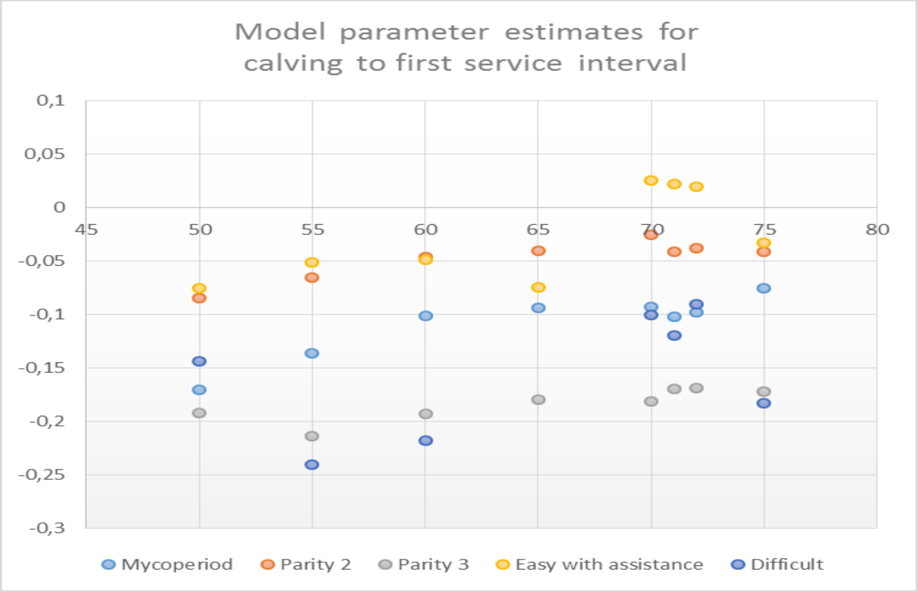

Figure 4
A plot of the parameter estimates (y-axis) with different break points for dichotomized calving to first service intervals.

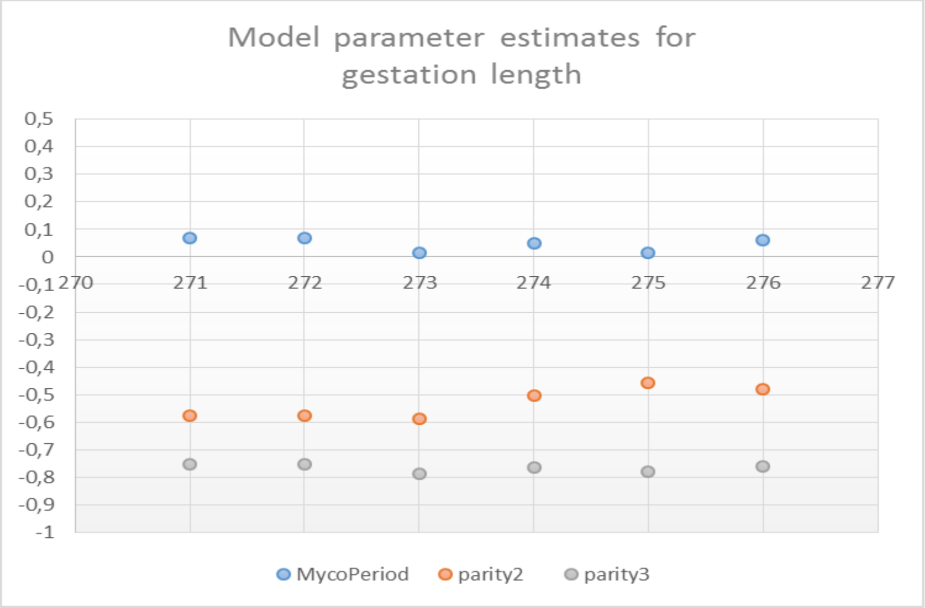

Figure 5
A plot of the parameter estimates (y-axis) with different breakpoints for dichotomized gestation lengths (271 to 276 days).

3.3 Model results

The results of the logistic model with CRFS as outcome have been converted into odds ratios (OR) to make them easier to interpret and compare. The model of CRFS showed no difference between the two periods, but old cows (parity 3+) had a higher chance of conceiving at first service. The calving difficulty level “difficult” resulted in a lower chance of conceiving at first service than calving difficulty level “easy without assistance” and “easy with assistance” (Table 7). The results are a herd average of 5 months, including only cows that calved.

A sensitivity test of the period chosen for comparison with the outbreak period in the model, i.e. comparing the outbreak period with a 5-month period before and after the original reference period one year before neither showed that the CRFS was different compared to the outbreak period.

The following interactions was tested: calving difficulty*parity ; parity*period ; period*calving difficulty. None of them were significant (p-value <0.05).

Table 7

Results according to the final multivariable logistic regression model for conception risk at first service (CRFS) in 59 Danish dairy herds that experienced a *Mycoplasma bovis* outbreak between 2010 and 2013. One herd did not register calving difficulty. The reference period is a 5-month period starting two months before the date one year before the outbreak start date. The table includes variance and standard deviation (Std) of the random effect and the parameter estimates, standard error (S.E.), odds ratios (OR) with 95% confidence interval (CI) and the p-value of the fixed effects.

Variables	Variance	Std		
Random effects				
Animal	0.04	0.19		
Herd	0.20	0.45		
	Parameter estimate	S.E.	OR (95% CI)	p-value
Intercept	-0.68	0.07		
Period				
Reference	0	0	1	
Outbreak	-0.05	0.043	0.95 (0.88 – 1.04)	0.28
Parity:				
1	0	0	1	
2	-0.03	0.05	0.97 (0.88 – 1.07)	0.56
3+	0.12	0.05	1.12 (1.02 – 1.24)	0.02
Calving difficulty:				
Easy without assistance	0	0	1	
Easy with assistance	-0.05	0.06	0.95 (0.84 – 1.07)	0.40
Difficult	-0.50	0.16	0.61 (0.44 – 0.83)	<0.01

The logistic model with binomial distribution of the CFS interval showed that cows in the outbreak period and old cows (parity 3+) had a larger risk of a CFS interval >71 days (Table 8). The results are a herd average of 5 months, including only cows that calved.

Table 8

Results according to the final multivariable logistic regression model for a dichotomized calving to first service interval (CFS) in 59 Danish dairy herds that experienced a *Mycoplasma bovis* outbreak between 2010 and 2013.The breakpoint is 71 days. One herd did not register calving difficulty. The reference period is a 5-month period starting two months before the date one year before the outbreak start date. The table includes variance and standard deviation (Std) of the random effect and the parameter estimates, standard error (S.E.), odds ratios (OR) with 95% confidence interval (CI) and the p-value of the fixed effects.

Variables	Variance	Std		
Random effects				
Animal	0.13	0.36		
Herd	0.51	0.72		
	Parameter estimate	S.E.	OR (95% CI)	p-value
Intercept	-0.20	0.10		
Period				
Reference	0	0	1	
Outbreak	0.10	0.04	1.11 (1.02 – 1.21)	0.02
Parity:				
1	0	0	1	
2	0.04	0.05	1.04 (0.94 - 1.16)	0.44
3+	0.17	0.05	1.18 (1.07 – 1.31)	<0.01
Calving difficulty:				
Easy without assistance	0	0	1	
Easy with assistance	-0.02	0.06	0.98 (0.86 -1.11)	0.72
Difficult	0.12	0.15	1.13 (0.85 -1.50)	0.41

The generalized linear model with gamma distribution of the CFS interval showed that cows in the outbreak period on average had 2.6 days longer CFS intervals compared to the same period the year before period. Parity 2 and 3+ cows had a longer CFS interval compared to first parity cows. Calving difficulty “easy with assistance” showed a small increase in mean number of days (Table 9). The results are a herd average of 5 months, including only cows that calved.

A sensitivity test of the two CFS models was performed, comparing the outbreak period with a 5-month period before (CP1) and after (CP2) the year before period. It indicated that the CFS was prolonged with 0.3 days for cows during the outbreak for the second model if comparing to CP1. If comparing to CP2 it showed a difference in mean of 7.8 days compared to the outbreak period. The results of the sensitivity test of the first model with binomial distribution of the CFS interval was consistent with the results of the original model.

The following interactions was tested: calving difficulty*parity ; parity*period ; period*calving difficulty. None of them were significant (p-value <0.05).

Table 9

Results according to the final multivariable mixed linear regression model (using a gamma distribution for estimation) for a continuous calving to first service interval (CFS) in 59 Danish dairy herds that experienced a *Mycoplasma bovis* outbreak between 2010 and 2013. One herd did not register calving difficulty. The reference period is a 5-month period starting two months before the date one year before the outbreak start date. The table includes variance and standard deviation (Std) of the random effect and residuals, and the parameter estimates, standard error (S.E.), mean – number of days with 95% confidence interval (CI) and the p-value of the fixed effects.

Variables	Variance	Std		
Random effects				
Animal	<0.01	<0.01		
Herd	<0.01	<0.01		
Residual	0.08	0.29		
	Parameter estimate	S.E.	Mean - number of days (95% CI)	p-value
Intercept (*Period:* Reference + *Parity*: 1 + *Calving difficulty*: Easy without assistance)	0.02	<0.01	53.6 (52.3 – 55.1)	<0.01
Period				
Outbreak	-0.00086	0.00008	56.2 (54.3 – 58.4)	<0.01
Parity:				
2	-0.00021	0.00011	54.3 (52.3 – 56.4)	0.05
3+	-0.00061	0.00015	55.5 (53.2 – 58.0)	<0.01
Calving difficulty:				
Easy with assistance	-0.00041	0.00014	54.9 (52.6 – 57.3)	<0.01
Difficult	0.00033	0.00033	52.7 (49.7 – 56.1)	0.32

Parity 2 and parity 3+ cows have a lower risk of a short gestation length (<272) compared to first parity cows. The risk of short gestation in the outbreak period was no different from the year before (Table 10). The results are a herd average of 3.5 months, including only cows that calved.

A sensitivity test of the models was performed, comparing the outbreak period with a 3.5 month period before and after the year before period. It supported the model results, showing a tendency of shorter GL during the outbreak.

The interaction of parity*period was tested but it was found non-significant (p-value <0.05).

Table 10

Results according to the final multivariable logistic regression model for a dichotomized gestation length (GL) in 60 Danish dairy herds that experienced a *Mycoplasma bovis* outbreak between 2010 and 2013. The breakpoint is 272 days. The reference period is a 3.5-month period starting 15 days before the date one year before the outbreak start date. The table includes variance and standard deviation (Std) of the random effect and the parameter estimates, standard error (S.E.), odds ratios (OR) with 95% confidence interval (CI) and the p-value of the fixed effects.

Variables	Variance	Std		
Random effects				
Animal	0.07	0.26		
Herd	0.32	0.56		
	Parameter estimate	S.E.	OR (95% CI)	p-value
Intercept	-2.17	0.12		
Period				
Reference	0	0		
Outbreak	0.06	0.07	1.07 (0.91 - 1.26)	0.40
Parity:				
1	0	0		
2	-0.56	0.08	0.56 (0.46 - 0.68)	<0.01
3+	-0.70	0.09	0.47 (0.38 - 0.58)	<0.01

4. Discussion

The study has analyzed the effect of a *M. bovis* outbreak on the reproduction of 60 Danish dairy herds. The herds have experienced an outbreak lasting at least three months in the years 2010 to 2014. Only one of the evaluated parameters showed a significant (p-value <0.05) effect of the outbreak period.

4.1 Conception risk at first service (CRFS)

The model of CRFS showed no difference between the two periods, but old cows (parity 3+) had a higher chance of conceiving at first service. The calving difficulty level “difficult” resulted in a lower chance of conceiving at first service

The result of CRFS analysis may indicate the pure fact that the fertility of the cows at their first insemination after calving is not affected by *M. bovis*. However, the lack of a detected association in this study may also be due to the uncertainty of the study design or that normal biological and management-related variation over time is large, which makes it difficult to demonstrate any differences between the periods with the available dataset.
Since we have asked the participating farmers if they have made any reproduction changes during the period we investigate, and then excluded the ones who had reported changes. I assume that the factors that affect CRFS had the same effect in our two periods.

In another study parity and seropositivity of *Mycobacterium avium* subspecies *paratuberculosis*, bovine leukemia virus (BLV) and bovine viral-diarrhea (BVD) were also found to have no effect on CRFS. In contrast, *Neospora caninum* seropositive cows were shown to have a lower risk of conceiving at first service in BVD seronegative herds compared to *Neospora caninum* seronegative herds (VanLeeuwen et al., 2010). A meta-analysis has shown that dystocia, stillbirth, milk fever, retained placenta, metritis, cystic ovaries, anestrus, ketosis, locomotion disorders and mastitis have a negative effect on CRFS (Fourichon et al., 2000). Due to the fact that *M. bovis* can cause mastitis it was expected that at least the herds that experienced mastitis as a clinical manifestation would have a change in CRFS. It is possible that it is because cows with clinical signs were culled almost immediately or there were too few cows with mastitis during the outbreak, to show an effect.

The decrease in CRFS for cows with calving difficulties is consistent with the results of (Fourichon et al., 2000). The increase in CRFS for old cows (parity 3+) might be because cows with low fertility are slaughtered as young cows.

Most of the diseases with an effect on the fertility are related to calving and the challenges of the postpartum cow with increasing milk production as an important risk factor. It is well known that these challenges affect the fertility (Butler, 2003).

It seems that contagious diseases do not affect CRFS significantly, while diseases such as milk fever, ketosis, retained placenta and mastitis have a larger effect. This might be due to the way the effect of contagious diseases are investigated. It is not only clinically ill animals that are included, but also animals that just have been tested antibody positive or all animals of a herd that has experienced an outbreak (VanLeeuwen et al., 2010). That does not say anything about whether the cows included ever have had clinical signs due to the contagious disease. Most of the diseases mentioned by Fourichon et al. (2000) are registered based on clinical signs.
Clinically ill cows due to *M. bovis* are most often culled in Denmark, and it is probably the reason why we do not see a change our study.

A meta-analysis has estimated that CRFS <40% is very poor and 46 – 54% is moderate (Fourichon et al., 2000). When comparing these estimates with the results of this study, it indicates that the included herds in general have a very poor CRFS. However, looking closer at the CRFS for each herd it indicates that some herds have a good CRFS (>55%), while others have a very poor CRFS. It is most likely because the herds where the farmer does not register pregnancy diagnosis for all of his cows, the cows that are slaughtered or culled are registered in our model as not having conceived at first service, even though they might have. Since we use the same way to calculate CRFS in both periods and we assume that the farmer has not changed management according to reproduction, it does not affect the result because the CRFS generally is low in both periods. If the farmer has culled many cows (as they often do when they have an outbreak) that did not have a pregnancy diagnosis it might affect the herd CRFS during the outbreak period. It would be a good idea to test if the number of culled cows affect the herd CRFS if using this model for another study.

4.2 Calving to first service (CFS)

The calculations of CFS showed a significant result in both ways of calculating the CFS in the outbreak period compared to the year before period. The logistic regression model with the binomial outcome showed that the cows in the outbreak period had an odds ratio of 1.1 for having a CFS >71 days compared to the cows in the year before group. Old cows (parity 3+) had an odds ratio of 1.18 for having a CFS >71 days compared to first parity cows. The logistic model with a continuous outcome showed that the cows in the outbreak period in average had a 2.6 days longer CFS and parity 3+ cows in average had a longer CFS too. The model also showed that cows with calving difficulty "easy with assistance" had a little prolonged CFS.

The sensitivity test of the model indicated that the CFS was prolonged with 0.3 days for cows during the outbreak for the second model if comparing to CP1. If comparing to CP2 it showed a difference in mean of 7.8 days compared to the outbreak period. The results of the sensitivity test of the first model with binomial distribution of the CFS interval was consistent with the results of the original model. One of the reasons why the reference period is exactly a year before the outbreak period is to avoid effects of for example season which has an effect on some reproduction parameters (Norman et al., 2009).

CFS has been used as a parameter to investigate the effect on the reproduction of bovine corona virus and bovine respiratory syncytial virus, based on milk samples and reproduction recordings. It showed that cows in antibody positive herds had more days from calving to first service compared to antibody negative herds, but it was not significant (Ohlson et al., 2010). CFS has been shown to be prolonged by dystocia, stillbirth, milk fever, retained placenta, metritis, cystic ovaries, anestrus, ketosis, and displaced abomasum (Fourichon et al., 2000). In this study only cows with calving difficulty "easy with assistance" had a prolonged CFS and not cows with difficult calvings as mentioned before. This is most likely because there only was 121 cows with difficult calvings in this study.

It seems that CFS is easily prolonged but it might be affected a lot by the farmer. If a herd has an outbreak, the farmer is likely to be stressed, and he might be more prone to push different tasks such as heat detection. E.g. if a farmer just has one cow in heat he do not want to spend time on

separating (and maybe inseminate) the cow because he has a lot of other things to do, and then she is inseminated 21 days later.
The reason why older cows (parity 2 and 3+) had a longer CFS interval might be because they in general have a higher yield than first parity cows, and high yield increases the risk of a longer CFS interval (Butler, 2003). The yield is generally increasing every year but I do not believe it accounts for that big a change in the CFS as the farmers most likely becomes a little bit better at controlling the negative energy balance of their cows every year as well.

4.3 Gestation length (GL)

Parity 2 and parity 3+ cows have a lower risk of a short gestation length (<272) compared to first parity cows. The risk of short gestation in the outbreak period was no different from the year before.

Earlier GL has been used to support late return to service rate, to estimate the abortion rate due to Bluetongue virus. They used GL from 175 days to 270 days and found an increase in short gestations during Bluetongue virus outbreaks (Nusinovici et al., 2012). This supports our study design of measuring short gestations because it is difficult to measure the true prevalence of abortions. Even though we do not cover all abortions we might catch some as short gestations. It could be argued that if GL <260 days was not changed to missing, more late abortions would have been included in the study, depending on how the farmer registered the abortion. Recording GL <260 as missing has been used before to minimize the risk of erroneous registrations (Norman et al., 2011).

It has previously been shown that older cows in general have a longer GL than primiparous cows, which is consistent with our results (Nogalski and Piwczynski, 2012).

4.4 General discussion

The way this subject was investigated has some advantages and disadvantages. Asking the farmers when they had an outbreak without having investigated any cows ourselves gives the study uncertainty about the exact time of the outbreak and if it truly was an outbreak. I have included all herds where the farmer answered that he had an outbreak or he had a few animals infected with *M. bovis*. This means that we do not know the exact proportion of infected or subclinical infected cows in the herd. Neither do we know the proportion of cows that was served by a bull, but I believe that it was a very small proportion.

A questionnaire does not give as detailed information of a herd as an interview would do but it would be more time-consuming if we had to interview all the farmers. An advantage is that we get in contact with many herds in a shorter time and thereby have a greater chance of finding significant tendencies.

If more time had been available, it could be interesting to change the study periods to shorter periods, which would give less cows but more certainty of the cows had met the bacteria. For the parameters, CFS and GL the herds with an outbreak start date after 30/07/2013 could have been included because the next calving date is not necessary for analyzing these parameters. By not including these herds, we have a more homogenous study population and the results of the parameters can be compared to each other more easily.

By looking at the estimates of the random effect of herd, it can be seen that some herds have experienced a considerably decrease in their reproduction results, while others did not have a change. The herds that have reported that they had a decrease in their reproduction results can be true, but it cannot be demonstrated that it is a general tendency for the herds who experienced an outbreak of *M. bovis*.

The parameters could have been analyzed by using other functions. Survival analysis has been used to analyze short gestations and linear regression has been used to analyze CFS (Ohlson et al., 2010; Nusinovici et al., 2012). If another model had been used for CFS, the proportion of cows that were never inseminated again could have been taken into account.

The sensitivity and specificity of the analysis would have been increased if blood samples or milk samples of individual animals were taken. The samples could be tested for *M. bovis* antibodies for example and the reproduction results of *M. bovis* positive animals and *M. bovis* negative animals in the same period could be compared.

When using the study design we do, taken into account that the bacteria is spread by contact and aerosols, we can see the impact of the bacteria being spread in the herd and what it causes on a herd level. This is more interesting to the farmer, because it has a larger economic impact to him than how a few of his cows may have worse reproduction results compared to the others.

In Estonia 2006 – 2008 they investigated the effect of *M. bovis* amongst other pathogens on insemination index (the number of inseminations used pr. pregnancy) and abortions, by using a questionnaire and serum samples. The found a low prevalence of *M. bovis* and no correlation to the two reproduction parameters (Raaperi et al., 2012).

In Denmark farmers tend to cull animals with clinical symptoms of a *M. bovis* infection. It has not been investigated if cows at a certain period of their gestation more often is infected than others. These animals are of course the ones the bacteria has the biggest impact on. They are culled almost immediately when the diagnosis is made and they have a very small chance of interfering the results of this study. It is most likely the effect on the cows without clinical disease that has been investigated in this study.

Since I want to compare the herds in this paper with themselves with just a year in between our two periods I find it reasonably to assume that a lot of factors have the same impact on the reproduction throughout the whole investigation period, like the environment and the genetics of the herd.
I am aware that the second period where I investigate the herd is during a disease outbreak and that the farmer and his employees most likely have changed the management and their point of focus intentionally or unintentionally. The degree of this change is in my opinion also dependent of the size and length of the outbreak, so that herds with just a couple of clinical cases have not really changed anything or made that big of an impact on the focus point of the farmer.

The results of this study can, to the best of my belief, be transferred to dairy herds in other western countries where they do not synchronize the cows, do use artificial insemination and where they have a climate and housing types that reminds of the Danish conditions.

5. Conclusion

The literature does not report any evidence that *M. bovis* affects the reproduction organs of the cow, besides some case rapports of abortions, and neither is there any evidence of an effect on the reproduction results.

The findings of this study demonstrated a small effect of a *M. bovis* outbreak on the reproduction results in a herd, more specifically a 2.6-day increase in the interval between calving and first service in cows that calved between two months before until three months after the farmer reported start of the outbreak.

This study did not investigate causal effects of the bacteria. We have investigated which changes an outbreak has caused in the reproduction results. We do not know if the changes in reproductive performance were directly due to *M. bovis* or if *M. bovis* had affected the cows' wellbeing in other ways, or if it was a result of the farmer's response to an outbreak.

It would take a more controlled study to investigate if the slightly prolonged CFS is due to *M. bovis* or if it is due to indirect effects of an outbreak.

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