Trace elements and cow health

The influence of cobalt, copper, selenium and zinc on the incidence of mastitis, endometritis and retentio secundinarum in Dutch dairy herds

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Content

Abstract

Mastitis, endometritis and retentio secundinarum are common health events in our current dairy cow and are responsible for high costs and decreased welfare of cows. Trace elements, in particular selenium, cobalt, copper and zinc, have become of particular interest of several authors in relation to these health events.

The aim of this study was to investigate whether there is an association between the incidence of mastitis, endometritis and retentio secundinarum on herd-level and the quantities of the trace elements cobalt, copper, selenium and zinc in the diet on herd-level.

In 2012 and 2013 the cases of mastitis, endometritis and retentio secundinarum of 22 Dutch dairy herds were collected and transformed into 6 dependent variables. The trace element content in the diet of 2012 was calculated and transformed into 18 independent variables, difference was made between the 4 trace elements, summer and winter period and lactation and dry period. Subsequently multivariate regression analysis was performed, with two significant ($p < 0,05$) models. An increased content of copper with 1% during summer >200 days into lactation, copper during summer in dry period and zinc during winter during lactation resulted in +0,017, -0,015 and -0,027 cases of mastitis per 10 cows in 2012 respectively ($p = 0,015$). An increased copper content in the diet of 1% resulted in -0,020 cases of mastitis, endometritis and retentio secundinarum in 2013 per 10 cows ($p = 0,041$).

Little variation was seen between the trace element content between the herds, most herds had above or far above advised levels of trace elements in the diet, this could be the result of the small study size. However, the results of this study provided us essential information about the trace element content in Dutch dairy herds and new comprehension for future studies.

Introduction

Background

Our current high yielding dairy cow is, due to genetic selection, able to produce a huge milk yield during lactation, which might be associated with health problems, especially during the periparturient period[1]. During this period the dairy cow is more susceptible for divers diseases like metabolic disorders, lameness, retentio secundinarum, clinical mastitis and endometritis[2] . Several of these diseases are linked to the diet fed to the cow during the early lactation and/or the dry period[3] . Trace elements, in particular selenium, cobalt, copper and zinc, in the diet of the dairy cows has become of particular interest of several authors in relation to some of the diseases mentioned above[4-8]. Andrieu *et al.* (2008) has suggested that trace element have significant influence on infertility, lameness and udder health[9]. The costs, vexation of the farmer, the shift of curative to preventive veterinary medicine, aimed reduction of antibiotic use and the animal welfare makes the prevention of these health events of interest.

Mastitis, lameness and subfertility problems represent the top 3 of health events of our current high yielding dairy cow[10,11]. Little evidence exist for a relationship between high milk yield and an increased risk of retentio secundinarum and metritis. However a relationship between the risk on mastitis and high milk yield seems to exist, probably due to the physiological stress experienced by cows during the first weeks of lactation[2]. Rapid acceleration of milk yield could be considered as an indicator for increased risk of health and fertility problems[2].

Health events

Mastitis

Mastitis, inflammation of the udder, is the most common disease in high yielding dairy herds and is responsible for high costs[10]. The incidence rate of mastitis varies between herds and depends on the exposure to pathogens, environmental and management factors and the immune status of the cow[10]. A study of Van den Borne *et al.* (2010) showed an incidence of 20,2% and 39,6% per 100 cow-years at risk for primiparous and multiparous cows respectively during the period of June 2004 and July 2005 in the Netherlands[12]. Barkema *et al.* (1999) showed an overall incidence rate in 274 herds of 0,263 cases/365 cow-days at risk[13].

The costs of mastitis varies between farms and countries and are due to reduced milk production, decreased milk quality, labor, treatment and applied drugs, discarded milk, veterinarian costs, culling and higher risk of other diseases[10,14,15]. The type of production system affects the costs, more intensive systems experience higher costs compared with less intensive systems[14]. Huijps *et al* (2008) developed a model based on 64 Dutch dairy herds to calculate the costs of mastitis. The costs of an individual case of clinical mastitis varies from €225 in the first month of lactation to €164 in the ninth month of lactation with an average cost of €210 per case[14].

Milk fever, retentio secundinarum, multiparity, winter season, SCC concentration in previous lactation are risk factors associated with increased mastitis incidence[10].

Retentio secundinarum

Retentio secundinarum is the condition in which the expulsion of the fetal membranes takes more than 24 hours. Usually the expulsion of the fetal membranes takes less than six to eight hours[16]. The definition of retentio secundinarum differs between authors, some suggest that retentio secundinarum exist after 12 hours others after 24 hours, these differences make comparison of studies more difficult. However, 95% of the cows with no retentio secundinarum after 24 hours had already expelled their fetal membranes after 12 hours, therefore the distinction between the two definitions is not of major importance. If retentio secundinarum exist, the mean duration of the condition is seven days[17].

Different incidence rates of retentio secundinarum are reported in literature, and the incidence rate is affected by the used definition. Incidence rates of 4-18%[16] and 3-39% of the parturitions are reported[18]. Opsomer *et al.* (2009) mentioned an incidence rate of 18% in 9 Belgium high yielding dairy herds[11].

Age, heredity, environment, hormones, twins, dystocia, stillbirths, abnormalities during the partus, gestation length and nutrition are predisposing factors for retention secundinarum [16,18,17,18]. Reduced uterine motility seems to play little or no role in the pathogenesis of retentio secundinarum. However an impaired leukocyte function to breakdown the connection between the caruncle and cotelydon seems to be the an important in the pathogenesis of retentio secundinarum[17]. Cows experiencing retentio secundinarum have a decreased number of leukocytes[3], and the leukocytes are less able to recognize the cotelydon tissue compared with leukocytes from cows not experiencing retentio secundinarum[18]. The impaired function and decreased numbers of leukocytes could explain how cows with retentio secundinarum have a three times higher risk of developing mastitis. There is not a causal connection between retentio secundinarum and mastitis, cows experiencing these health events share a depressed immune function which makes them more susceptible for health problems[3].

Besides mastitis, cows experiencing retentio secundinarum are at higher risk for developing ketosis, milk fever, displacement of abomasum, vulvar discharge and endometritis[16,17,19]. Opsomer *et al.* (2009) reported that cows with retentio secundinarum are 6 times more at risk to develop endometritis[11], other authors reported that 25-50% of the cows with retentio secundinarum develop endometritis[17] and it has negative impact on reproduction[16,19] . The pregnancy rate in cows with retentio secundinarum is reduced by 15% compared with cows not experiencing retentio secundinarum[17] and the calving to first service and calving to conception interval is prolonged with 7 and 18 days ($p < 0,05$) respectively[16].

Kossaibati *et al.* (1997) calculated, based on 90 Holstein-Friesian dairy herds in England during 1992/1993 with an average milk yield of 6000L, that the direct costs of a case of retentio secundinarum was £83,25, mainly due to reduced milk yield. The overall costs were calculated at £298,29, mainly due to longer calving interval, increased risk of culling and increased vulvar discharge of[19].

Endometritis

Endometritis and metritis are important postpartum health events, both associated with prolonged calving to conception interval and reduced pregnancy rate[17]. Discussion about the definition of these health events exist, probably due to the diagnostic method used to determine the illness[11]. Metritis is inflammation of the endometrium, the glandular layer and muscular layer of the uterus within 7 days postpartum and is characterized by systemic signs of illness, fever, uterine discharge, anorexia and decrease in production. Endometritis is inflammation of the endometrium and glandular layer of the uterus from 3 week postpartum without systemic sings of illness, but with (muco-) purulent discharge[17,20]. Both diseases have similar etiology and may predispose each other[20]. The most important predisposing factor for (endo)metritis is retentio secundinarum, but difficulties during calving, dystocia, stillbirth, twin births, primiparity, winter season, male calves and factors that reduce feed intake and impair the immunity of the cow are also risk factors for (endo)metritis[10,11,17].

Cows with (endo)metritis have 2 times more risk of developing ketosis, which makes cows more susceptible for left displaced abomasum and cystic ovarian disease[11].

The costs of endometritis varies per case between €160-420[11] and are caused by treatment costs, reduced milk yield, labor, culling and longer calving to conception interval[17].

Incidence rates of endometritis differs between various studies. Opsomer *et al.* (2009) mention an incidence rate of acute (endo)metritis of 15% in 9 Belgium high yielding dairy herds and that the incidence rate of uterine diseases in high yielding cows has increased over time[11]. LeBlanc *et al.* (2008) reported an incidence rate of 15-20% and 30-35% of (endo)metritis at 4 to 6 and 4 to 9 weeks postpartum respectively[17].

Trace elements

Fifteen different trace elements, arsenic, chromium, cobalt, copper, fluorine, iodine, iron, manganese, molybdenum, nickel, selenium, silicon, tin, vanadium and zinc, less than 0,01% of the total body mass, are identified for maintaining normal mammalian biological body function. Eight of these trace elements are considered to be essential for cattle. Trace elements serve various functions in the body. They can act as cofactor for enzymes, or serve as an essential part of the structure of enzymes. Other trace elements play a role in the normal function of hormones or vitamins[21,22] .

Deficiencies of some trace elements can result in reduced growth, fertility and milk production or impaired immune function which makes the cow more susceptible for divers diseases. These symptoms may exist without showing particular clinical signs of the deficiency[22,23]. Trace element deficiency can either be primary or secondary. Low concentration of trace elements in the diet may result in primary deficiency. Secondary trace mineral deficiency can be the result of an excess of other minerals in the diet and infectious or parasitic diseases, circumstances that reduces the bioavailability of the trace element[21,22].

The trace element content in the forage depends on the trace mineral composition and availability of trace elements in the soil. The origin of the rock, glaciation, surface erosion, salinization, use of pesticides, fertilizers, manure and sludge, surrounding industry and transportation are factors affecting the composition of the soil[21]. pH, moisture, other elements and microbial activity have influence on the uptake of trace elements in the soil by crops. Soil with alkaline pH show an increased biological availability of selenium and molybdenum. In soils with acid pH selenium is less available, but copper availability is increased[21]. Maturation of crops cause a decline in some trace elements, especially copper and zinc[21]. The recommended trace element content in the diet depends on various factors such as milk yield and lactation stage[24].

Cobalt

Cobalt is an essential part of vitamin B_{12}, which is produced in the rumen of cattle and sheep and is required for the enzymes methionine synthase and methylmalonyl CoA mutase. The latter is important for the production of glucose from propionate[22]. Vitamin B_{12} is necessary for the growth of several ruminal microorganisms. Consequently low cobalt availability may result in an impaired fermentation. Vitamin B_{12} is stored in the liver, cows fed sufficient cobalt have enough vitamin B_{12} reserves for several months if the cobalt content in the diet suddenly becomes insufficient[22]. The first signs of cobalt deficiency are reduced appetite and reduced growth or weight loss. If the deficiency continues, cows show severe weight loss, pale skin and mucous membranes, pica, nervousness and higher susceptibility for diseases[22,24].

In a paper of MacPherson (1989) it is suggested that the immune response of cattle is affected by cobalt deficiency, with a reduction of 50 per cent in the number of neutrophils[25]. Wintergerst *et al.* (2007) has investigated the importance of vitamin B_{12}. They suggest that a deficiency of this vitamin is essential to maintain an adequate immune status, as a deficiency may result in a reduction of lymphocytes, natural killer cells and CD8+ cells[26]. Several studies support these negative effects of cobalt deficiency, which result in higher disease susceptibility. Cobalt deficient sheep showed a higher incidence of respiratory and enteric infections and a higher susceptibility for parasitic infections compared with cobalt sufficient sheep. In an experiment conduced with cobalt deficient and cobalt sufficient fed sheep, the cobalt deficient sheep showed 18,2% mortality compared with 0% in de sufficient group after receiving L3 Ostertagia circumcinta larves 3 times per week for 16 weeks. The cobalt deficient sheep showed more plasma pepsinogen and fecal eggs counts. In other experiments, cobalt deficient sheep showed less antibody production after vaccination compared with cobalt sufficient sheep. Neutrophils from cobalt deficient calves showed at least 50% reduction killing percentage of *Candida albicans* compared with neutrophils from cobalt sufficient fed calves[25]. Cobalt deficient calves showed a shorter prepatent period after experimentally being infected with Ostertagia ostertagi compared with cobalt sufficient calves; 26 vs 41 days[27].

The CVB (2010) recommended levels of cobalt in the diet during the dry period is 1,1 mg and 1,2 mg per day during the last three weeks and 8-3 weeks prior to calving respectively. During lactation the cobalt demand increases and the advised levels rise towards 1,9 mg and 2,4 mg per day for 20 kg and 40 kg milk yield respectively[24].

Copper

Copper is part of several enzymes, like cytochrome oxidase, superoxide dismutase (SOD), lysyl oxidase, tyrosinase, ceruloplasmin, which are necessary for energy metabolism, detoxification of oxygen radicals, pigmentation, the structure of collagen and elastine and iron transport[22] . Copper is stored in the liver[28] and absorbed in the intestines. Cattle with a fully developed rumen are less able to absorb copper compared with young calves with an undeveloped rumen. Molybdenum and sulfur in the diet are probably responsible for the reduced copper absorption, by the formation of thiomolybdates in the rumen, which interact with copper in the intestines forming insoluble complexes which are poorly absorbed. Absorbed copper-thiomolybdate complexes binds tightly to albumin in blood plasma, making the copper unavailable for biochemical interactions[22] . To prevent the formation of copper-thiomolybdate complexes, the molybdenum:copper ratio in the feed should not exceed 1:2[21] . Sulfur is also able to interact with copper without molybdenum, probably by forming copper sulfide bonds in the intestines. These complexes are insoluble and cannot be absorbed. Raising the dietary sulfur concentration from 0,1 till 0,3-0,4% reduces copper uptake by 30-40%[22] .

Zinc[21] and iron in the feed may also reduce the copper availability[22,25] . Even small quantities of iron reduce the uptake. The source of iron could be soil contaminated feed, groundwater[21] or to the diet added mineral supplements. Mineral supplements should not contain more than 10.000 mg/kg iron[25]. Owing to the interactions, the content of copper in the diet is less value when the concentrations of sulfur, molybdenum and iron is unknown[22].

In a paper of Failla *et al.* (2003) is it suggested that copper has important functions considering the immune system. Copper deficiency decreases the synthesis of Interleukin-2 which serves an important function in the activation of immune cells[23]. Spears (2000) reported in a review that the phagocytic, cell-mediated and humoral activity of the immune system is affected by copper deficiency[7]. These adverse effect on the immune system result in higher disease susceptibility. MacPherson (1989) has reported that a low plasma copper status resulted in increased microbial infections in lambs under field conditions. *In vitro* tests showed that the capacity of immune cells to kill *Candida albicans* is reduced under copper deficient circumstances[25].

The earliest sign of copper deficiency is depigmentation of the hair and a rough coat[22] . Anemia, due to decreased production of ceruloplasmin and ferroxidase II which results in reduced iron release form reticuloendothelial cells and therefore cause impaired erythrocyte production and integrity is another sign of copper deficiency . Heart failure, due to reduced cytochrome oxidase level and therefore myocardial degeneration is also associated with copper deficiency[21]. Calves with severe copper deficiency are stiff and lame and could experience spontaneous fractures, due to a decrease in amine of lysyl oxidase which are important for the collagen structure of the bone[21] .
Elevated copper plasma levels are seen in animals experiencing chronic stress or infections and are usually not the result of high dietary copper[4,21].

The CVB (2010) recommended levels of copper in the diet during the dry period is 277 mg per day, the amount of copper during the lactation depends on the milk yield, 227 mg or 260 mg per day for a milk yield of 20 kg and 40 kg respectively[24].

Selenium
Selenium, a component of several enzymes such as glutathionperoxidase (GSH-Px), has important functions in the cell cytosol maintaining low concentrations of reactive oxygen species (ROS), which can damage the polyunsaturated fatty acids of the cell membrane, DNA and other enzymes in the cell[6] . Selenium and vitamin E are associated with each other, vitamin E is part of the cell membrane and protects the membrane against ROS[6] . ROS are formed during normal cell functions and in higher quantities during periods of higher metabolic activity, for instance parturition[8,29] .
Immune cells are highly sensitive for ROS because of their high content of polyunsaturated fatty acids in the cell membrane and production of ROS during phagocytosis[8,30] . The formed ROS is converted by SOD into one oxygen and one hydrogen peroxide molecule. These hydrogen peroxide molecules are converted into water molecules. A low concentration of selenium in the diet can cause a low activity of GSH-Px in the cell, causing an accumulation of hydrogen peroxide. High levels of hydrogen peroxide in polymorph nuclear neutrophils (PMN) are associated with lower bacterial killing [6].
Selenium has influence on both the innate and adaptive immune system. Neutrophils from selenium deficient cattle are able to ingest pathogens, *in vitro*, but the ability to kill those pathogens is reduced compared with neutrophils obtained from selenium sufficient cattle. This reduced pathogen killing ability is associated with decreased GSH-Px activity in the cell cytosol, the ROS formed during phagocytosis destroys the neutrophil[30].

The absorption of selenium in ruminants is lower compared with non-ruminants, probably due to the formation of insoluble selenium form in the rumen. Bioavailability of selenium is affected by numerous factors, including the sulfur content in the diet, type of diet, calcium content in the diet and form of selenium. Organic selenium in yeast (selenomethionine) appears to have better bioavailability compared with selenite (inorganic)[22,31]. Not only has selenomethinine better bioavailability, but it is 40% more effective in increasing GSH-Px activity compared with selenite[31] .

White muscle disease in calves is probably the best known symptom of selenium deficiency[21,25]. But clinical mastitis[1,6,8] , retained fetal membranes[1,6,8,25]and endometritis[1,6,25] are also linked to selenium deficiency, probably as a result of an impaired immune function.

The CVB (2010) recommended levels of selenium during the dry period is 1,44 mg per day. During lactation the advised levels of selenium in the diet increase to 2,72 mg and 4,22 mg for 20 kg and 40 kg milk yield per day respectively[24].

Zinc

Zinc is part of over 70 metalloenzymes including, carbonic anhydrase, DNA and RNA polymerase, alcohol dehydrogenase, copper- zinc superoxide dismutase and alkaline phosphatase[22] and plays an important role in protein synthesis and nucleic acid metabolism[21]. The absorption of zinc takes place in the abomasum and small intestine High calcium levels in the diet reduce zinc absorption and proteins from plant seeds requires higher zinc levels in the diet[21,22]. Organic and inorganic forms of zinc seems to have similar bioavailability, however zinc excretion in zinc deficient lambs fed inorganic zinc was higher compared with organic fed lambs[22,31]. Stress and inflammation cause redistribution of zinc which results in low zinc plasma levels[4,21,22].

Zinc plays a role during the wound healing process and therefore is import for the first line of defense. Zinc deficiencies, caused by an genetic dysfunction which inhibits the intestinal zinc absorption, may lead to dysfunction of the cell-mediated immunity[21] and low antibody response[4] . In a study of Enjalbert *et al* (2006) low zinc plasma status was associated with increased incidence of mastitis, endometritis and retentio secundinarum[4]. Zinc is an essential part of the keratin plug in the teat canal[9] , which is part of the physical barrier of the innate immune defense of the udder[1] . Like cobalt and selenium deficient animals, zinc deficient lambs were, less able to kill *Candida albicans* [25]. In humans marginal zinc deficiencies results in lower disease resistance and immune responses, however in cattle a marginal deficiency does not result in malfunctions of the cell-mediated or humoral immune system[7] .

Zinc deficient cattle show reduced feed intake, reduced growth, salvation, swollen hocks and knees, stiff gait, parakeratotic lesion on the neck, head, and around the nostrils and claws, dermatitis of the teat skin, infectious pododermatitis, failure in wound healing, and loss of hair[21,22].

During the dry period the CVB (2010) recommended levels of zinc in the diet is 246 mg. During lactation the amount of zinc in the diet is 490 mg and 763 mg for 20 kg and 40 kg milk yield respectively[24].

Aim of the study

Following the results of previous studies, it is clear that trace elements may affect the immunity of cattle and have influence on the incidence of numerous health events. Therefore the aim of the present study is to investigate whether there is an association between the incidence of mastitis, endometritis and retentio secundinarum on herd-level and the quantities of the trace elements cobalt, copper, selenium and zinc in the diet on herd-level under current Dutch conditions. Besides the individual effects of these trace elements, the aim of this study is also to investigate if these trace elements influence each other effect considering cow health.

Materials and methods

Study design

During 2012 and 2013 twenty-nine Dutch dairy herds, distributed through the Netherlands, with Holstein-Friesian cows participating in the “Weerbaar Vee” (Resilient Livestock) project collected data concerning animal health events of cows > 22 months. The number of events and cows affected with clinical mastitis, endometritis, retentio secundinarum, metabolic disorders, claw diseases and airway diseases were diagnosed and kept in a database by the farmer and were finally collected by the project group.

From twenty-three of these herds, the trace element content of the diet fed in 2012 was calculated. The trace element concentration in the data set of one of the dairy herds was disarranged with another herd and consequently eliminated from the study, leaving 22 herds available for analysis.

Owing to the small sample size, the conducted study could be considered as a pilot study to investigate whether there are differences between herds regarding the trace element content in the diet and disease incidence.

Data

The diets fed during the period of January till December 2012 to the twenty-three dairy herds participating in the project were collected using a questionnaire and analyzed by BLGG AgroXpertus. The trace element content was calculated by the Nutrient Management Institute Wageningen (NMI). This was limited to these four trace elements: cobalt, copper, selenium and zinc. Distinction between the trace element content in the diet was made for primiparous and multiparous cows, winter and summer period, dry period and lactation period. The lactation period was split into 4 stages:

- 0 - 14 days into lactation,
- 15 - 100 days into lactation,
- 101 - 200 days into lactation,
- > 200 days into lactation.

This resulted in twenty different diets per trace element per herd.

Trace elements
The trace element content of the diet, was expressed as percentages of the levels advised by CVB according to the “Mineraal Wijzer”. The percentage of each trace element was, according to table 1 classified into four groups.

Table 1: Distinction between the four different groups of trace elements content in the diet expressed towards the CVB advised levels.

	Percentage	Group
Under advised level:	0-99%	1
Advised level:	100-150%	2
Over advised level:	151-250%	3
Far over advised level	>250%	4

The trace element content in the diet of the herds participating in the project were expressed towards CVB advised levels and categorized into four groups.

Initially eighty different independent variables were calculated (see attachment 5). This number of independent variables were too large for statistical analysis. Therefore new independent variables were formed by combining the initial independent variables. The different trace elements, the winter and summer period and lactation and dry period were kept separate, so that the effect of the trace element content in the diet on cow health during these periods could be observed in the results.
The regrouping of the trace elements was performed using the previous determined trace element groups, i.e. group 1, 2, 3 or 4. If little variation in trace element category was observed within a herd between the different lactation stages and/or between multiparous and primiparous cows, these groups were combined.

Cobalt, selenium and zinc, were each transformed into four independent variables, with separation between the lactation and dry period en winter and summer period. The copper content in the diet showed more variation within the herds between different locational stages, this resulted into six different independent variables with a separation in the lactation period: < 200 days into lactation and > 200 days into lactation (seen attachment 5).
The lactation subgroups were combined using the following formula:

Average trace element content in the diet of the herd =

$$\frac{(0\text{-}14 \text{ days}) + (15\text{-}100 \text{ days}) + (100\text{-}200 \text{ days}) + (>200 \text{ days})}{4}$$

No distinction between primiparous and multiparous cows was made in the new independent variables, as these two groups in most herds were not represented in equal numbers, the calculation of the new independent variables was performed with the following formula:

Average trace element content in the diet of the herd =

$$\frac{(2 \text{ x multiparous trace element content}) + (1 \text{ x primiparous trace element content})}{3}$$

Disease incidence
Health events concerning clinical mastitis, endometritis and retentio secundinarum used in this study, were monitored by the farmers.

The definition used in this study for retention secundinarum is: failure to expel the fetal membranes within 24 hours.

In this study we aimed to measure the effect of trace elements on the postpartum uterine health. Therefore no distinction was made between endometritis and metritis, the term endometritis is used for both health events.

The total cases of the three individual experienced health events during a year were combined resulting in the dependent variable ‘disease incidence’ per herd. In order to use this variable to compare herds, it was transformed into the variable ‘disease incidence per 10 cows’, using the following formula:

Disease incidence per 10 cows =

Total number of these health events / Total number of cows present in the herd * 10

The variable disease incidence per 10 cows was used instead of the percentage of affected cows per herds, hence most herds counted less than hundred cows.

The variable disease incidence consisted out of the cases of clinical mastitis, endometritis and retentio secundinarum during a year. The distribution of the three separate health events within the variable disease incidence was calculated as a percentage of the total disease incidence. Owing to the results of this calculation, two additional variables were formed using a similar formula as shown above: ‘mastitis incidence per 10 cows’ and ‘retentio secundinarum and endometritis incidence per 10 cows’. In total six variables, three for each year were formed.

Categorization of disease incidence
For further analysis and to compare the herds, the herds were classified into three groups: low (1), medium (2) or high (3) disease, mastitis or retentio secundinarum and endometritis incidence. Herds with an incidence below the lower quartile (25% of the herds) were considered as low incidence herds, above the upper quartile (25% of the herds) as high incidence herds and herds within the interquartile range (50% of the herds) as medium incidence herds.

Statistical methods

Statistical analysis was performed using SPSS 22. Initially descriptives of the variables, histograms and scatterplots of quantitative variables and bar charts of qualitative variables were made.

Univariate regression analysis.
For each of the six dependent variables an univariate regression analysis with each of the eighteen independent variables was performed. Independent variables with regression coefficients with a p-value <0,20 were used in the multivariate backwards regression analysis after verifying the underlying assumptions .

Assumption one: the relationship between x and y is linear.
An imaginary line in scatterplot of the dependent and independent variable should be drawn.

Assumption two: the residuals are independent.
For this assumption a histogram of the standardized residuals was drawn. The residuals should be distributed normally with a mean of zero. Residuals that are not normally distributed indicate heteroscedasticity.

Assumption three: the residuals are distributed normally with constant variance.
A scatterplot with on the x-axis the standardized predicted value and on the y-axis the standardized residuals was drawn to verify this assumption. If this assumption is satisfied, the scatter of the residuals is random. A certain pattern in the scatterplot could be an indication of non-linearity, heteroscedasticity or both.

The regression coefficients, p-values, and assumptions were presented in a table. The relatively high p-value in the univariate regression analysis, was chosen due to the small sample size of the study. Outliers in a small sample size have more impact on the outcome compared with outliers in a large sample size. When choosing a lower p-value, and therefore excluding independent variables for multivariate regression analysis, the interaction they may have on other independent variables, which could lead to significant influence on the dependent variable could be missed.

Multivariate regression analysis
The limited study size would normally not be large enough to perform multivariate regression analysis, however multivariate regression analysis is conducted considering this study being a pilot study.

The estimated regression line is represented by the following equation:

$$Y = a + b_1x_1 + b_2x_2 + b_3 + x_3 \ldots + b_kx_k$$

Y is the dependent variable
a is a constant term
b are the regression coefficients corresponding to k independent, x, independent variables.

Collinearity
In multivariate regression analysis we may expect a association between independent variables. The effect they have on each other could strengthen or weaken the individual influence they have on the dependent variable. However extremely correlated independent variables may result in collinearity, which makes the predicted regression coefficients unreliable. Before conducting the multivariate regression analysis, the independent variables were examined for correlation among these independent variables. Pearson's correlation coefficient was used for normal distributed variables and Spearman's rank correlation coefficient was used for not normal distributed variables.
Variables correlated $r > 0,6$ were not used in the same model. The variable with the most significant regression coefficient obtained from the univariate regression model was used in the multivariate regression model. If both variables had approximately the same p-value, the variable with the highest regression coefficient obtained from the univariate regression (negative or positive) was used in the model.

Results

Herds

Twenty-two dairy herds with a mean of 78 (SD ± 25) dairy cows in 2012 and 87 (SD ± 24) dairy cows in 2013 were used for statistical analysis.

Disease incidence

The number of health events which occurred in 2012 and 2013 in the herds used in this study are represented in table 2 and 3.
Mastitis was responsible for the major part in the health events in both 2012 and 2013. Therefore, the dependent variable disease incidence per 10 cows was split into two new dependent variables: 'mastitis incidence per 10 cows' and 'endometritis and retentio secundinarum incidence per 10 cows'. With this separation, the influence of the trace elements on the different health events was more distinct. Histograms showing the distribution of the three independent variables are added in attachment 2.

Table 2: Health events which occurred in 2012 in the herds participating in the study.

2012	Number of cows	Percentage
Mastitis	722	65%
Retentio secundinarum	121	11%
Endometritis	266	24%
Total number of health events	1109	100%

In total 1109 health events occurred in 2012, mastitis represented 65% of the health events, retentio secundinarum represented 11% of the health events and endometritis represented 24% of the health events. One cow could have experienced multiple health events during the year. The total number of cows > twenty-two months participating in the study in 2012 was 1718.

Table 3: Health events which occurred in 2013 in the herds participating in the study.

2013	Number of animals	Percentage
Mastitis	603	67%
Retentio secundinarum	112	13%
Endometritis	182	20%
Total number of health events	897	100%

In total 897 health events occurred in 2013, mastitis represented 67% of the health events, retentio secundinarum represented 13% of the health events and endometritis represented 20% of the health events. One cow could have experienced multiple health events during the year. The total number of cows > twenty-two months participating in the study in 2013 was 1913.

The herds were categorized into group 1, 2 or 3 according to the disease incidence of the herd (see table 4). Histograms of the dependent variables showed no normal distribution of the data (see attachment 2). Not normal distributed variables can be categorized by using the lower and upper quartile of the cumulative frequency[32]. When using the quartiles as a categorization method, outliers have no impact on the categorization of the herds, which is of particular interest in small sample sizes.

Table 4: Disease incidence per 10 cows in 2012 and 2013 and the incidence group to which the herds belong.

Disease incidence	2012	2013	Group	N
Low	0-4,80	0-3,26	1	5
Medium	4,81-7,63	3,27-6,53	2	12
High	7,64-10	6,54-10	3	5

The categorization of the herds according to their disease incidence per 10 cows was performed by using the upper and lower quartile of the disease incidence of all the herds participating in this study. Herds with a disease incidence per 10 cows below the lower quartile was considered as low incidence herds (group 1) and herds with a disease incidence above the upper quartile were considered as high incidence herds (group 3). Herds within the interquartile range were considered as medium incidence herds (group 2).

Tables of the categorization of the remaining dependent variables, mastitis incidence and endometritis and retentio secundinarum incidence, bar charts of the distribution of the herds between the disease incidence categories are added in attachment 3.

Trace elements

Eighteen different independent variables were used in the regression analysis. Below the description of each of the independent variables.

Cobalt

Lactation winter period: Every herd participating in this study had more cobalt in the diet compared with recommended levels. The mean content of cobalt in the diet during this period was 525% (± 268%) towards advised levels. The trace element content of eighteen of the twenty-two herds belonged to category four, the remaining herds belonged to category three, little variety between the herds existed.

Dry winter period: Like during the lactation period, little variety between herds existed. The mean content of cobalt in the diet during this period was 409% (± 170%) towards advised levels. One herd had a below advised level of cobalt in the diet, but the remaining herds belonged either to category 3 (four herds) or category 4 (seventeen herds).
Lactation summer period: Slightly more variety was seen in the diet during this period compared with the lactation period during winter season. The mean cobalt content of the diet was 461% (± 268%) towards advised levels.Category one and two contained both one herd. However the majority of the herds belonged to category three (four herds) or category four (sixteen herds).

Dry summer period: This period has much resemblance compared with the dry period during winter season. The mean cobalt content of the diet was 363% (± 155%) towards advised levels. One herd had a below advised level of cobalt content in the diet, the remaining herds belonged to category three (five herds) or category four (sixteen herds).

Copper

<200 days winter period: During this period little variation between herds existed. Every herd had above advised levels of copper in the diet with a mean of 275% (± 57%). Nine of the herds belonged to category three and thirteen herds belonged to category four.

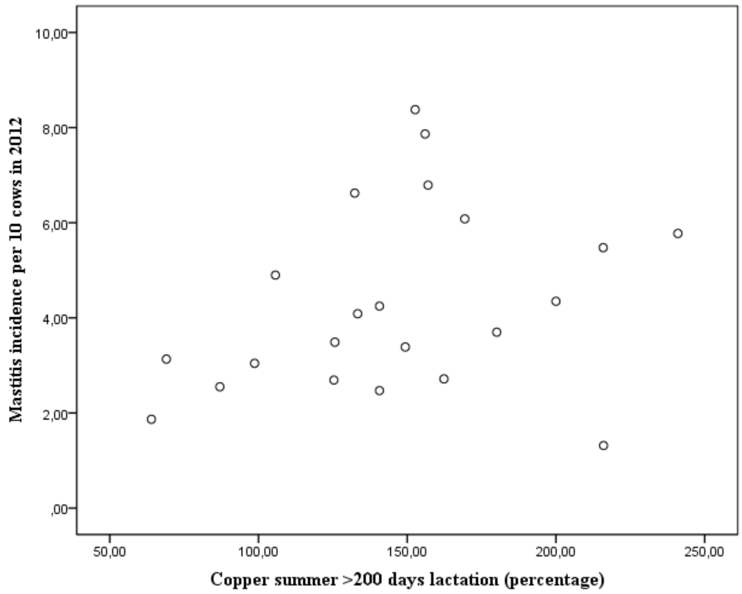
>200 days winter period: Compared with the first two-hundred days of lactation, more variation existed between herds during the remaining days of the lactation. The mean copper content in the diet was 193% (± 45%) towards advised levels. Category two was represented by four herds, category three by sixteen herds and category four by two herds.

Dry winter period: During this period, more variation between the herds existed. The mean copper content in the diet was 167% (± 73%) towards advised levels. Six herds belonged to the first category (under advised level), three herds belonged to the second category, eleven herds belonged to the third category and two herds belonged to category four.

<200 days summer period: Like during the same period in the winter season not much variation between the herds existed. The mean copper content in the diet was 213% (± 57%) towards advised levels. Category one and two each contained one herd, most herds belonged to category three (fourteen herds) and category four was represented by six herds.

>200 days summer period: This period showed more variation between the herds. Remarkably none of the herds belonged to category four, this resulted in a mean copper content of 146% (± 46%) towards advised levels. Most herds belonged to category three (ten herds). Eight herds had advised levels of copper in the diet (category two) and four herds had below advised level in the diet (category one). The variation in the copper content between the herds is represented in figure 1. In this scatterplot with on the x-axis the copper content in the diet and on the y-axis the mastitis incidence per 10 cows in 2012, an imaginary line with positive coefficient could be drawn.

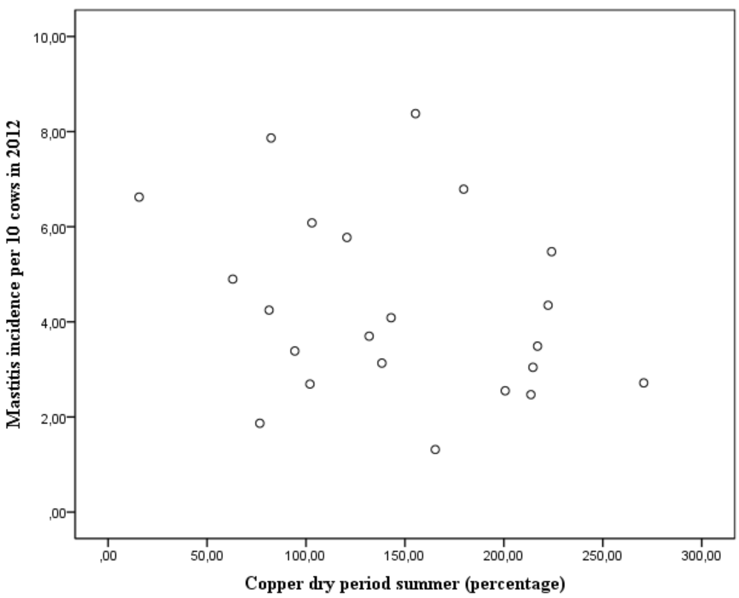
Figure 1: Scatterplot with on the x-axis the copper content in the diet > 200 days lactation during the summer period and on the y-axis the mastitis incidence per 10 cows in 2012.



Each point represents one herd (n=22). The x-axis represents the copper content in the diet >200 days lactation during summer period. The y-axis represents the mastitis incidence per 10 cows in 2012. An imaginary line with positive coefficient could be drawn, indicating that higher levels of copper in the diet during this period results in higher mastitis incidence.

Dry summer period: Like during the previous period, the copper content in the feed during this period showed more variation and each category was represented. The mean copper content in the diet of 146% (± 66%) towards advised levels. Category one contained six herds, category two also six herds, category three nine herds and category four one herd. In figure 2 the variation between the herds concerning the copper content in the diet during the dry period in the summer is represented by a scatterplot.

Figure 2: Scatterplot with on the x-axis the copper content in the diet during the dry period during the summer and on the y-axis the mastitis incidence per 10 cows in 2012.



Each point represents one herd (n=22). The x-axis represents the copper content in the diet during the dry period during summer. The y-axis represents the mastitis incidence per 10 cows in 2012. An imaginary line with negative coefficient could be drawn, indicating that higher levels of copper in the diet during this period results in lower mastitis incidence.

Selenium

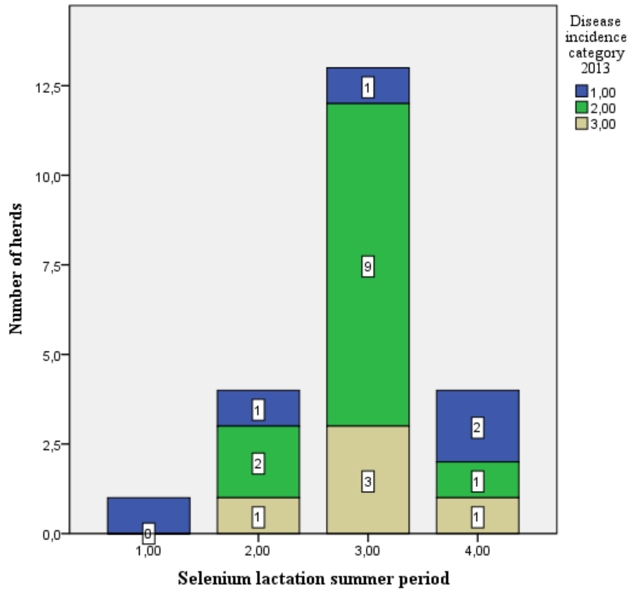
Lactation winter period: During this period most herds had more than advised levels of selenium in the diet. The mean selenium content in the diet was 220% (± 45%) towards advised levels. Fourteen herds belonged to category two and six herds to category four. Two herds had advised levels of selenium in the diet (category two) and none of the herds belonged to category one.

Dry winter period: During the dry period in the winter, more variation existed compared with the lactation period. Owing to the large proportion of the herds feeding far above advised levels, the mean selenium content was 261% (± 113%) towards advised levels. Category one was represented by two herds, category two also by two herds, category three by six herds and category four by twelve herds.

Lactation summer period: The selenium content in the diet during lactation in the summer period did show variation between herds. One herd belonged to category one and four herds belonged to category two. Most herds had above advised levels of selenium in the diet (thirteen herds) or far above advised levels (four herds), which resulted in a mean selenium content of 200% (± 53%) towards advised levels.

Figure 3 represents the distribution of the categories of the variable "disease incidence per 10 cows in 2013" between the categories of selenium in the diet. No tendency of less disease by higher levels of selenium is seen or vice versa. Actually, the herd with the least amount of selenium in the diet belongs to disease incidence category 1, however two herds of disease incidence category 1 also belongs to the highest selenium category.

Figure 3: Distribution of "disease incidence per 10 cows in 2013" between the categories of selenium in the diet.



This bar chart represent the selenium content in the diet during the lactation during summer period and the disease incidence in 2013. The selenium content in the diet was separated into 4 categories, 1: under advised level, 2: advised level, 3: above advised level, 4: far above advised level. The bars are subdivided into 3 disease incidence categories, 1: low incidence, 2: medium incidence, 3: high incidence. The number inside the bars represent the number of herds belonging to a similar disease incidence group. No relationship between the selenium content in the diet and disease incidence is observed.

Dry summer period: Like the selenium content of the diet in the dry period during winter season, most herds during summer season belonged to category four (ten herds) and category three (eight herds). The remaining herds belonged to category two (one herd) and category one (three herds). The mean selenium content in the diet was 242% (±101%) towards advised levels.

Zinc

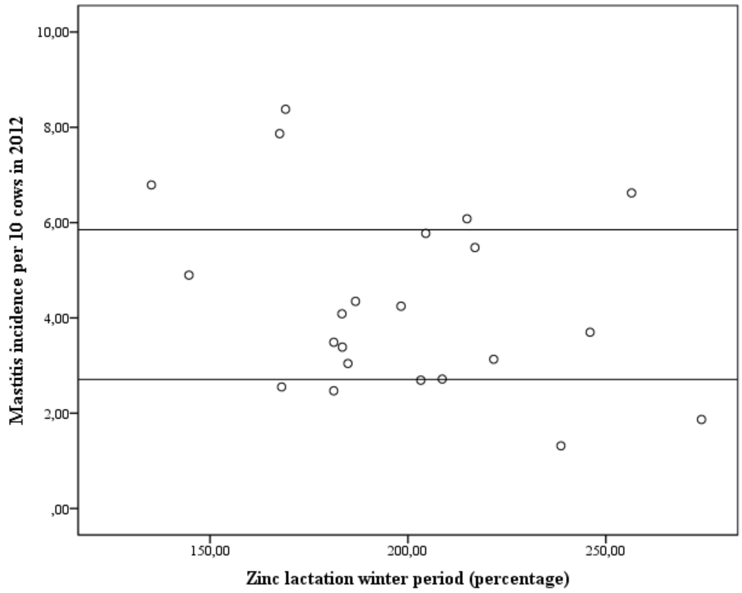
Lactation winter period: Most herds, eighteen in total, during this period belonged to category three. The remaining herds belonged to category two (two herds) and four (two herds). None of the herds belonged two category one. The mean zinc content in the diet was 199% (± 35%) towards advised levels.

Dry winter period: Compared with the lactation, even less variation between herds was seen during the dry period. The mean zinc content in the diet during this period was 310 % (± 70%) towards advised levels. The majority of the herds, sixteen, belonged to category four, the remaining six herds to category three.

Lactation summer period: The variety of zinc content in the diet between herds during this period was somehow similar compared with zinc lactation during the winter season. Also eighteen herds belonged to category three, the remaining four herds belonged to category two, resulting in a mean zinc content in the diet of 178% (± 33%) towards advised levels.

Figure 4 shows a scatterplot of zinc lactation during winter period and mastitis incidence per 10 cows in 2012. The horizontal lines divides the scatter plot in high incidence herds (above upper horizontal line) and low incidence herds (under lower horizontal line). High incidence herds are more seen on the left side of the x-axis (less trace element in the diet) and low incidence herds more on the right side of the x-axis (higher trace element content in the diet), indicating lower incidence by higher trace element content of the feed. Some other zinc variables do show to some extent a similar, but not significant, picture.

Figure 4: Scatterplot with the amount of zinc in the diet during the lactation in the winter period and the mastitis incidence per 10 cows in 2012.



Each point represent one herd (n=22) The horizontal lines separates the herds into high incidence herds (above upper line) or low incidence herds (under lower line). An imaginary line with negative regression coefficient could be drawn, indicating that during the lactation period in the winter season more zinc in the diet results in less cases of mastitis.

Dry summer period: Not much variation between herds existed during this period. Most herds belonged to category four, nineteen herds in total. The mean zinc content in the diet was 308% (±77%) towards advised levels. Two herds belonged to category three and one herd to category two.

Regression analysis

Linear regression was performed, to determine the relationship between the trace element content in the diet and disease incidence of the herds.

Table 5 shows the result of the univariate regression analysis with dependent variable disease incidence per 10 cows in 2012. Two independent variables had $p < 0,20$ and were used in the multivariate regression analysis. The distribution of the residuals were normal and the scatter of the standardized predicted values and standardized residuals were random. The distribution of both independent variables was normal. Therefore Pearson's correlation to calculated correlation between the variables was performed. No significant correlation existed (r = 0,025 with p = 0,912). Multivariate regression did not result in a significant model (p = 0,105).

Table 5: Result of the univariate regression analysis with dependent variable disease incidence per 10 cows in 2012.

Independent variable	Regression coefficient	P-value
Copper <200 days summer period	0,010	0,165
Zinc lactation winter period	-0,017	0,129

The dependent variable used in this univariate regression analysis was disease incidence per 10 cows in 2012. Two independent variables with p-value <0,20 were used in a multivariate regression model, which did not result in a significant model.

Table 6 shows the results of the univariate regression analysis with dependent variable mastitis incidence per 10 cows in 2012. Four independent variables had p < 0,20 and were used in the multivariate regression analysis. The distribution of the residuals were all normal and the scatterplot of the predicted values and standardized residuals did not show any patron.

Table 6: Result of the univariate regression analysis with dependent variable mastitis incidence per 10 cows in 2012.

Independent variable	Regression coefficient	P-value
Copper dry winter period	-0,008	0,183
Copper >200 days summer period	0,012	0,184
Copper dry summer period	-0,009	0,185
Zinc lactation winter period	-0,019	0,126

The dependent variable used in this univariate regression analysis was mastitis incidence per 10 cows in 2012. Four independent variables had p-value <0,20 and could, depending on any collinearity, be used in a multivariate regression model.

Before performing multivariate regression analysis, Pearson's correlation coefficient between the independent variables was calculated. Significant (p < 0,01) collinearity existed between the independent variables copper in the dry period during the winter period and copper in the dry period during the summer period. Therefore one of these two variables was eliminated from the multivariate regression model.
The independent variable with the most significant regression coefficient in the univariate regression, copper dry during winter period (B = -0,008 p = 0,183) versus copper dry during summer period (B = -0,009 p = 0,185), was used in the multivariate regression model. The outcome model was significant (p = 0,034) with adjusted R^2 of 0,271, however the regression coefficient of copper lactation >200 days during summer period was not significant (p = 0,073). Exchanging copper dry during winter period for copper dry during summer period in the model resulted in a more significant model (p = 0,015) and higher adjusted R^2 (0,337). Every independent variable in this regression model had a significant (p < 0,05) regression coefficient (see table 7).

Table 7: Result of the multivariate regression analysis with dependent variable mastitis incidence per 10 cows in 2012.

Independent variable	Regression coefficient	P-value
Copper >200 days lactation summer period.	0.017	0.035
Copper dry during summer period.	-0.015	0.016
Zinc lactation during winter period.	-0.027	0.018

Initially four independent variables with p-value<0,20 were entered in the model with dependent variable mastitis incidence per 10 cows in 2012, due to collinearity copper dry during winter period was excluded from the model.

Table 8 shows the results of the univariate regression analysis with dependent variable endometritis and retentio secundinarum incidence per 10 cows in 2012. Two independent variables had p < 0,20 and depending on the assumptions could be used for multivariate regression analysis. The residuals of cobalt dry during winter period were not normally distributed, this could be an indication for heteroscedasticity or non-linearity, the scatterplot of the predicted values and standardized residuals did not show any patron.
Both independent variables were distributed normally, the Pearson's correlation coefficient resulted in r = 0,438 with p = 0,041. Even though the correlation between the variables was significant, the r was below the chosen value (r > 0,6) for excluding variables from the multivariate regression analysis.
The multivariate regression analysis did not result in a significant model (p = 0,266).

Table 8: Result of the univariate regression analysis with dependent variable endometritis and retentio secundinarum incidence per 10 cows in 2012.

Independent variable	Regression coefficient	P-value
Cobalt dry winter period	0,002	0,174
Copper dry summer period	0,006	0,159

The dependent variable used in the univariate regression analysis was retentio secundinarum and endometritis incidence per 10 cows in 2012. Two independent variables with p-value <0,20 were used in a multivariate regression model.

Table 9 shows the result of the univariate regression analysis with dependent variable disease incidence per 10 cows in 2013. Solely copper >200 days during winter period had a significant outcome p < 0,05 with regression coefficient -0,020. Regarding the distribution of the residuals and the scatterplot of the predicted values and standardized residuals, there is no indication for non-linearity or heteroscedasticity.

Table 9: Result of the univariate regression analysis with dependent variable disease incidence per 10 cows in 2013.

Independent variable	Regression coefficient	P-value
Copper >200 days winter period	-0,020	0,041

The dependent variable used in the univariate regression analysis was disease incidence per 10 cows in 2013. Only one independent variable had p-value <0,20, therefore multivariate regression analysis for disease incidence per 10 cows in 2013 was not conducted.

Table 10 shows the outcome of the univariate regression with dependent variable mastitis incidence per 10 cows in 2013. Four independent variables have p < 0,20 and could be used for multivariate regression analysis. The residuals of the four variables were distributed normally and the scatter of the predicted values and standardized residuals was random, indicating linearity and homoscedasticity. The distribution of the four independent variables was normal.

Table 10: Result of the univariate regression analysis with dependent variable mastitis incidence per 10 cows in 2013.

Independent variable	Regression coefficient	P-value
Copper >200 days winter period	-0,013	0,064
Copper dry winter period	-0,006	0,170
Selenium dry winter period	-0,004	0,106
Selenium dry summer period	-0,005	0,100

The dependent variable used in the univariate regression analysis was mastitis incidence per 10 cows in 2013. Four independent variables had p-value <0,20 and could, depending on any collinearity, be used in a multivariate regression model.

Pearson's correlation coefficient shows significant ($p < 0,01$) correlation between multiple independent variables:
- Copper dry during winter period with selenium dry during winter period (r = 0,842),
- Copper dry during winter period with selenium dry during summer period (r = 0,847),
- Selenium dry during winter period with selenium dry during summer period (r = 0,913).

Owing to collinearity between the above independent variables, a multivariate regression model with these independent variables could not be performed. As copper >200 days during winter period was not correlated with any of the other independent variables, three multivariate regression models were performed, none of which significant (see table 11).

Table 11: Results of the multivariate regression models with dependent variable mastitis incidence per 10 cows in 2013.

Independent variable 1	Independent variable 2	p-value
copper > 200 days winter period	copper dry winter period	0,062
copper > 200 days winter period	selenium dry during summer period	0,077
copper > 200 days winter period	selenium dry during winter period	0,072

Initially four independent variables were selected for the multivariate model based upon the results of the univariate regression (regression coefficient with p-value<0,20). Collinearity between several independent variables resulted in excluding variables from multivariate regression. This resulted into 3 models, none of which significant.

Table 12 shows the result of the univariate regression analysis with dependent variable endometritis and retentio secundinarum incidence per 10 cows in 2013. Cobalt lactation during summer period is distributed to the right due to two herds feeding 1218% and 878% toward advised levels, but copper >200 days during winter period was normally distributed. The Pearson's coefficient of the variables was r = 0,081 with $p > 0,05$. The scatterplot of the standardized residuals and the predicted values did not show any patron, the residuals of cobalt lactation during summer period were not normally distributed, indicating heteroscedasticity or non-linearity.
The multivariate regression did not produce a significant ($p = 0,254$) model

Table 12: Result of the univariate regression analysis with dependent variable endometritis and retentio secundinarum incidence per 10 cows in 2013.

Independent variable	Regression coefficient	P-value
Cobalt lactation summer period	0,001	0,173
Copper >200 days winter period	-0,007	0,164

The dependent variable used in this univariate regression analysis was endometritis and retentio secundinarum incidence per 10 cows in 2013. Two independent variables with p-value <0,20 were used in a multivariate regression model.

Owing to the limited data in this study, not all the residuals in the univariate regression analysis were distributed normally. However the residuals of the variables used in the multivariate regression analysis were distributed normally, and the residuals of the significant model were also distributed normally.

Interpretation of the multivariate regression analysis

Mastitis incidence per 10 cows in 2012

$Y= 9,281 + 0,017x_1 - 0,015x_2 - 0,027x_3$

Where:
x_1 is the copper content in the feed in the lactation period over 200 days during the summer period,
x_2 the copper content in the feed during the dry period during the winter period,
x_3 the zinc content of the feed during lactation during winter period.

The adjusted R^2 of the model is 0,337.

Every 1% increase of copper content in the diet during lactation in the period of 200 days during the summer period, every 1% increase of copper content in the feed during the dry period during the summer period and every 1% increase of zinc content in the feed during the lactation during the winter period, resulted in an increase of 0,017 and decrease of 0,015 and 0,027 cases of mastitis per 10 cows in 2012 respectively.

Disease incidence per 10 cows in 2013.

$Y= 8,792 - 0,020x$

Where x is the copper content of the feed in the lactation period over 200 days during the winter period.

The adjusted R^2 of the model is 0,153.

Every 1% increase of copper content in the feed during the lactation from 200 days until the dry period during the winter period led to a reduction of 0,020 cases per 10 cows in 2013, i.e. 0,020 cows less affected with mastitis, endometritis or retentio secundinarum per 10 cows present in the herd.

Discussion

The aim of the study was to investigate whether there is an association between the incidence of mastitis, endometritis and retentio secundinarum on herd-level and the quantities of the trace elements cobalt, copper, selenium and zinc in the diet on herd-level. According to the above results, in the present study there was an association between the copper and zinc content in the diet and the mastitis incidence in 2012 and the copper content in the diet and the disease incidence in 2013.

The present study showed that an 1% increased copper content in the diet during the dry period during summer period and 1% increased zinc content during lactation during winter period resulted in a decreased mastitis incidence of 0,015 and 0,027 cases per 10 cows in 2012 respectively. This study also showed us that an 1% increased copper content in the diet during lactation >200 days during the summer period resulted in an increased mastitis incidence of 0,017 cases per 10 cows in 2012.
The disease incidence in 2013 in this study decreased with 0,020 cases per 10 cows by an 1% increased copper content in the diet during lactation >200 days during winter period.

Strengths and limitations

Health events
As mentioned earlier in this paper, the three health events investigated in this study probably share a common factor which affects the immune status and makes the cow more susceptible for health problems. Trace elements have effect on the immune status of the cow. Therefore, these three health events were combined as one variable representing the immune status of the cow.

Study size
The limited study size (22 herds), could have been the cause for little variation between herds. Especially for zinc, cobalt and selenium this could be an explanation for the few significant results. The under advised levels group was not comprehensively represented in the latter two trace elements and the content of zinc in the diet in none of the herds was below advised levels.

Trace elements in the diet
The result in this study suggests that the trace element content in the diet of Dutch herds contain on average more than advised levels and problems due to low trace element levels in the diet do not play a major role in Dutch herds. However, the diet concentrations of the trace elements used in the present study are calculated based upon information about the diet given by the farmer, the concentrations are not actual measured in the diet. Calculation instead of measuring the trace elements in the diet could have resulted into differences between the concentrations used in this study and the actual concentrations that were fed.

Trace elements in plasma
No information about the actual plasma levels of the trace elements were available, these plasma levels have effect on the biochemical processes of the trace elements in the body. The bioavailability of trace elements in the diet could be affected by other elements in the diet, type of diet or source of the trace element, i.e. organic or inorganic[6,21,22,31]. Some herds had low trace element level in the diet during the dry period, however the liver or kidney contain

enough stored trace element to maintain normal plasma levels if the diet is low in trace elements during several weeks or months.

Farmers

The farmers participating in this study represented a group that is willing to spend much time and effort towards obtaining excellent cow health, this makes the participating herds in this study not necessarily representative for the average herd in the Netherlands.

The data concerning health events were collected by the farmers, any missed, wrongly diagnosed or inadequate treated cases could have, resulted in lower of higher numbers of health events and therefore could have had influence on the dependent variable and final outcome. Any (accidental) changes made in the diet could have had influence on the independent variable and finale outcome. Feeding trace elements as powder-supplements is subjected to human error. It is possible that the amount of trace elements calculated does not match the actual amount of trace element given, due to under- or overestimation of the amount of the supplement, or due to carelessness by the farmer. Additional, the trace element content of 2012 was calculated and compared with the disease incidence in 2012 and 2013. No information about the trace element content in the diet during 2013 was available. Recalculation of the trace element content in the diet off 2013 could have resulted in other results, since much variation exist between different silages. It is not likely that trace elements fed during the beginning of 2012 still affect the cow health in 2013, however the analysis should be interpreted is this manner. Although trace elements are stored in the liver and during periods of low concentration in the diet may act as reserve. This could explain why herds with low trace element content in the diet did not show high disease incidence, as the plasma levels remain adequate due to the trace elements stored in body tissue.

Influence of confounders

Any other (metabolic) diseases, infections or other stressors, for instance the weather, overcrowding, hygiene, management of dry and start-up period, could enhance the disease incidence and could have had influence on the final outcome of the analysis.

In our analysis we did not take the interval calving - mastitis appearance into account. Most health disorders occur during the transition period[33]. Information about the time of the mastitis cases could have provide us essential information concerning the interpretation of the results. As different factors could affect the mastitis susceptibility during different lactation stages.

The health events of primiparous and multiparous cows in the present study were added together, while the health events they experience could have different etiology and the incidence rate of the health events between both age groups usually differs. The mineral plasma status of these two groups could also be different, as primiparous cows usually do not receive concentrates before calving. Separation between both age groups could have given us more accurate results, and more information on the influence of the trace elements on both groups.

Initially, the purpose of the study was solely to investigate the relationship between the trace elements and the variable “disease incidence”, representing mastitis, endometritis and retentio secundinarum. Due to the high proportion of mastitis within the variable disease incidence (65% in 2012 and 67% in 2013, see attachment 1), I separated mastitis as one variable for more clear results. Otherwise the cases of mastitis would have had much impact in the variable disease incidence on the other health events and the effect of the trace elements on

the cases of endometritis and retentio secundinarum would get overshadowed. Even though, separation of the variable "endometritis and retentio secundinarum incidence" into two variables could have given us even more accurate results.
Cows experiencing retention secundinarum are more likely to develop endometritis[11,17]. The cases of endometritis, which have developed out of retentio secundinarum used in this study are considered as two independent cases. This aggravates the endometritis and retention secundinarum incidence in the herds. It would have been better to separate the cases of endometritis which were a result from retention secundinarum from the cases of endometritis without retention secundinarum history.

Strengths
The study provides us essential information about the trace element content in the diet in Dutch dairy herds. It showed us that the content of zinc and cobalt in the diet in most herds was above or far above advised levels, and that most herds fed sufficient selenium towards recommended levels during the lactation, but some variation during the dry period was found. However, most variety between herds was seen in the copper content in the diet, especially during the dry period and during the summer period.
The results in the present study suggest that feeding trace elements above advised levels does not necessarily result in improved cow health. Apart from the four independent variables which did have significant effect on the mastitis and disease incidence in 2012 and 2013 respectively, the remaining independent variables did not show similar results. Therefore, feeding trace elements above advised levels is only necessary if a shortage is established through blood analysis, or when clinical signs are highly suggesting the shortage of a particular trace element.

Most remarkable is the conflicting effect of copper on the mastitis incidence, depending on the lactation period it was either associated with higher or lower mastitis incidence. This showed us that the division of the trace elements in summer and winter period and lactation and dry period is important for the interpretation of the results but also raises more questions about the influence of trace elements and any confounders on cow health. However these results have given us useful input for future studies.

The multivariate regression analysis showed us that independent trace elements amplify each other effect on the mastitis incidence in 2012, i.e. the regression coefficients of the individual independent variables increased in the multivariate regression model compared with the regression coefficients of the same independent variables in the univariate regression analysis.

Finally, the study design of the present study was on herd-level. Most studies performed concerning trace element and cow health are on cow-level or under controlled circumstances with significant results. We showed that a portion of the variance in mastitis incidence in 2012 and disease incidence in 2013 could be explained by the trace element content in the diet. However the remaining portion of the variance is due to other factors. Thus, numerous other factors are involved concerning cow health. Supplementation of trace elements is not always the best solution to prevent disease, especially when plasma levels are unknown. Supplementation should be based on plasma levels and requirements of the cows.

In a study of Scaletti *et al.* 2003 twenty-eight heifers were separated in two groups. Both groups were fed identical diets apart from the copper content. One group received from 60 days antepartum until 42 days postpartum a copper deficient diet, another group received

during the same period a copper sufficient diet. At day 34 postpartum one pathogen free quarter was experimentally challenged with *E.coli*. The copper sufficient cows had less bacterial positive quarters at day fourteen ($p < 0,05$), but higher prevalence of coagulase negative staphylococci at day 14 ($p < 0,03$) and 24 hours ($p < 0,03$) pre challenge.
The udder of the cows were scored on a five point scale. Normal udders with normal milk were scored as 1 and a swollen udders, with abnormal milk and systemic signs of infection were scored as 5. Copper sufficient cows showed lower udder scores at 24 hours post challenge but the copper deficient cows showed lower udder scores 144 hours post challenge[34]. The study design of Scaletti *et al.* does not match our study design. However in the study of Scaletti *et al.* higher copper content in the diet did not result in an unanimous lower mastitis incidence, which reconcile with the outcome in the present study. As in the present study, in the study of Scaletti *et al.* no information about the actual plasma levels were known. Therefore, copper deficient fed cows probably could, due to liver storages, still remain copper sufficient plasma levels.

No relationship between the selenium content in the diet and disease incidence could be established in the present study. In an a paper of Spears *et al.* (2011) selenium supplementation of diets low in selenium were associated with decreased retentio secundinarum and metritis incidence and low selenium was related with higher mastitis incidence[22]. In this study we did not observe similar results, probably due to the limited herds with low selenium in the diet.

In a study of Smith *et al.* (1984) four groups of eighty cows in total received either a selenium and vitamin E deficient diet, a selenium and vitamin E sufficient diet or a diet with either of one sufficient and the other deficient. The results showed that cows supplemented with selenium and vitamin E or exclusively vitamin E had 37% reduction of clinical cases compared with the controls. Supplementation of exclusively selenium showed 12% reduction compared with the controls[5]. These results suggest that the selenium status has little effect on the mastitis incidence, but the combination of both selenium and vitamin E has much effect on the mastitis incidence. Similar results were obtained in a study by Weiss *et al.* (1997)[35]. The results of these two studies show the relationship between selenium and vitamin E, and that the supplementation of one element depends on the amount of the other element or vitamin in the diet[8]. In the present study we did not have information about the vitamin E content in the diet, which could explain the obtained results. As seen in Figure 3 on page 18 no tendency of less disease by higher levels of selenium is seen or vice versa. Possibly different vitamin E status of the herds are responsible for the inconsistent results.

Andrieu *et al.* (2008), suggest that adequate levels of trace elements may protect cows against mastitis. Supplementation of organic copper, zinc and selenium resulted in improved udder health and lowered somatic cell count (SCC) compared with cows supplemented with inorganic forms of the same trace elements. Bioavailability of organic forms was better compared with inorganic forms. However, the importance of the management is also mentioned in this article in relation with the prevention of mastitis[9]. The results from this study is important to understand the outcome of the present study. No information was available about the sources, i.e. organic or inorganic, of the trace elements fed in the various diets. Therefore, high trace element content in the diet, doesn't necessarily result in adequate plasma and tissue levels, responsible for the biochemical processes in the body.

In a study of Cook *et al.* (2007) 425 cows were split into two groups. One group received at drying off a bolus with 500 mg selenium, 3400 mg iodine and 350 mg cobalt, the other did not receive any treatment. The treatment group showed significant (p = 0,02) reduced incidence of retentio secundinarum compared with the control group[29]. In our study no significant effect of any of the trace elements on the incidence of retentio secundinarum and endometritis was found. However, two major differences between these studies exist. First, in our study the cases of endometritis and retentio secundinarum were added together and we had no information about the iodine content of the diet. In the same paper, the importance of iodine in relation to retentio secundinarum and the relationship of selenium to transform T_4 into the metabolic active T_3 is suggested.

Enjalbert *et al.* (2006) found in their study a relationship between low zinc plasma level and increased risk for mastitis, retentio secundinarum and endometritis, and an increased risk of retentio secundinarum in herds with low selenium plasma levels[4]. In the present study, the plasma levels were unknown, but based on the outcome of the study of Enjalbert *et al.* information about the actual plasma levels could have provide us essential information, concerning the relation between the intake and plasma levels. Eventually, trace element deficiency in plasma result in clinical signs or impaired immune system. The bioavailability of trace elements are affected by numerous factors, therefore a sufficient trace element content in the diet does not necessarily result in adequate plasma levels.

In a review of Hemingway (1999), it is suggested that intake of selenium and vitamin E above advised levels at the start of the dry period could significantly reduce the incidence of clinical mastitis[36]. In the present study higher levels of selenium in the diet did not result in lower incidence of mastitis compared with lower levels of selenium in the diet. However, the diet of the herds participating in the present study had during the whole lactation adequate, above or far above advised levels in their diet, not much difference in dietary selenium intake consisted during the lactation or dry period. A few herds had under advised levels of selenium in their diet during the dry period, this was not associated with higher incidence of mastitis. An explanation could be the limited herds with low selenium in the diet during the dry period and the multifactorial nature of this health event.

Clinical interpretation

Copper

Compared with other trace elements, the copper content in the diet shoed the most variation between the herds. In most periods, each category is represented by one or more herds. Most variation was found during the dry period in both the winter and summer season followed by the lactation >200 days. Copper was the only trace element with $p < 0,20$ for each dependent variable in the univariate regression and could be used in each model of the multivariate regression, probably due to the variation between herds.

The outcome of the multivariate regression with higher mastitis incidence in 2012 by elevated copper level in the diet during summer season during lactation >200 days and lower mastitis incidence in 2012 by elevated copper levels during summer period during dry period is remarkable and was not expected. How could an elevated level of copper in the diet at the end of lactation result in higher mastitis incidence, but is a few weeks later responsible for lower mastitis incidence? Perhaps besides copper itself, other factors belonging to the source of the copper may be responsible for the higher mastitis incidence. A plausible explanation could be that another element simultaneously fed with the copper source is responsible for the higher mastitis incidence and copper acts as a proxy for that not measured element.

According to the CVB 2010, the average copper content in summer is 8,1 mg/kgDM which is below the need of a dry or cow in lactation. The content of grass silage and corn silage is 7,7 mg/kgDM and 3,8 mg/kgDM respectively and even more below the need of a dry and lactating cow[24]. Therefore the main source of copper in the diet are concentrates and mineral supplements. For good interpretation of the results it is essential to know what the copper source is, i.e. concentrates, mineral supplements, boluses or added powder over the feed. A high copper content in the diet due to feeding concentrates at the end of the lactation could result in an energy rich diet which could result in an increased body condition score (BCS). High body condition (≥4 on a 5 point scale) score ante partum is a predisposing factor for metabolic diseases postpartum, which are predisposing factors for mastitis[2]. However high milk yield is also a predisposing factor for mastitis[2]. High yielding cows need, on average, more concentrates compared with lower yielding cows. Thus, maybe a high copper content in the diet does not increase the mastitis incidence, but the milk yield increases mastitis incidence and copper intake serves as a proxy for milk yield. However this hypothesis does not answer the question why this was only found during the summer period and not during winter period.

Otherwise, a high copper level in the dry period could be a proxy for other elements in the diet having beneficiary effect on the mastitis incidence. For instance cows receiving high levels of copper in the dry period could also consume high level of magnesium in the same supplement. Elevated levels of magnesium during the dry period could prevent milk fever peripartum, which is a predisposing factor for mastitis[37]. Similar situation could also increase vitamin E in the diet, which is usually low in stored forages[6,38], probably cows receiving high levels of copper also receive high levels of vitamin E. The effect of vitamin E on the incidence of mastitis is reported is numerous articles[5,38]. Since elevated copper content in the diet during the winter period, when stored forages are fed, was associated with lower mastitis incidence, increased vitamin E could have been the actual cause for the decline in mastitis incidence.

Cobalt

No relationship between the disease incidence and cobalt content in the diet of these herds was found. Little variation of cobalt content in the diet between herds exist and most herds had above or far above advised levels of cobalt content in the diet both during the winter and the summer season and both during lactation and dry period. This could explain why no significant relationship between the cobalt content in the diet and the disease incidence exist, even though the analysis was performed quantitatively. This suggests that raising the cobalt content in the diet far above advised levels does not improve cow health. Any consequences of low cobalt levels in the diet could not have been estimates. A study with more herds, consequently more data, could result in a different outcome, assuming more variety between the data exist.

Selenium

Despite the variety in the selenium content of the diet between the herds, no significant results were produced in the multivariate regression analysis. Even though in this study these selenium variables have no significant effect on the outcome, it is possible that these variables do have effect on the dependent and become significant in a study with larger sample size.

Several authors report the effect of selenium on health events[5,8,38], but no similar results were obtained in this study, probably due to the sufficient levels of selenium in most diets in the present study. This shows that increasing the selenium content in the diet above sufficient levels does not always result in improved cow health. Actually, excessive levels of selenium in the diet could result in decreased cow health.

Zinc
Zinc during lactation in the winter season is the only zinc variable used in multivariate regression analysis, with mastitis incidence in 2012 as dependent. None of the other zinc variables had $p < 0{,}20$ and could therefore not used in the multivariate regression analysis. The limited data and lack of variation of zinc content in the diet between the herds is probably the reason why regression analysis did deliver only one significant result. Most herds feed above or far above advised levels, especially during the dry period. None of the herds had under advised level of zinc in the feed during any of the periods. The zinc content of roughages normally meet the zinc demands of the cow[24].

Future studies

The present study has provided use with useful information about the relation between trace elements and disease incidence, however many questions remain. Especially concerning the conflicting influence of copper on the disease incidence. Other factors associated with elevated copper content in the diet, like amount of concentrates or supplements should further be investigated, to figure out what factor is actual responsible for the remarkable result.

For future studies, the plasma level of the different trace elements could provide essential information. Another analysis together with the content of the trace elements in the diet could give us information about the resource of trace elements in plasma in relation with the content in the diet.

In future studies separation of the endometritis and retentio secundinarum cases could provide more valuable information, as no separation in the data is made between a stand-alone case of endometritis and endometritis which resulted out of retentio secundinarum.

Conclusion

The aim of this study was to investigate the relationship between the cobalt, copper, selenium and zinc content in the diet and the incidence of mastitis, endometritis and retentio secundinarum in twenty-two Dutch dairy herds. The results of this study showed that in most herds the trace element content in the diet was above or far above CVB advised levels and that the copper content in the diet showed most variation between herds. This trace element was significant associated with the mastitis incidence in 2012. Depending on the lactation stage, increased levels of copper in the diet either resulted in higher or lower mastitis incidence. An increased zinc content in the diet fed during lactation during winter period resulted in significant lower mastitis incidence in 2012.
Cobalt and selenium did not have significant influence on the disease incidence, probably due to the limited variation between herds concerning the content of these trace elements in the diet.

Based upon the results of this study, increasing the trace element content in the diet above advised levels does not always result in significant lower disease incidence. However, the study size in the present study was small, a larger study size with more variation between herds concerning trace element content in the diet could result in a different outcome. Future studies with more herds and additional information about the resource of the trace elements could help us to answer the questions raised during this study.

References

1. Sordillo LM. Factors affecting mammary gland immunity and mastitis susceptibility. *Livest Prod Sci*. 2005;98(1-2):89-99.

2. Ingvartsen KL, Moyes K. Nutrition, immune function and health of dairy cattle. *Animal*. 2013;7(SUPPL.1):112-122.

3. Overton TR, Waldron MR. Nutritional management of transition dairy cows: Strategies to optimize metabolic health. *J Dairy Sci*. 2004;87(SUPPL. 1):E105-E119.

4. Enjalbert F, Lebreton P, Salat O. Effects of copper, zinc and selenium status on performance and health in commercial dairy and beef herds: Retrospective study. *J Anim Physiol Anim Nutr*. 2006;90(11-12):459-466.

5. Smith KL, Harrison JH, Hancock DD, Todhunter DA, Conrad HR. Effect of vitamin E and selenium supplementation on incidence of clinical mastitis and duration of clinical symptoms. *J Dairy Sci*. 1984;67(6):1293-1300.

6. Smith KL, Hogan JS, Weiss WP. Dietary vitamin E and selenium affect mastitis and milk quality. *J Anim Sci*. 1997;75(6):1659-1665. Accessed 24 January 2014.

7. Spears JW. Micronutrients and immune function in cattle. *Proc Nutr Soc*. 2000;59(4):587-594.

8. Spears JW, Weiss WP. Role of antioxidants and trace elements in health and immunity of transition dairy cows. *Vet J*. 2008;176(1):70-76.

9. Andrieu S. Is there a role for organic trace element supplements in transition cow health? *Vet J*. 2008;176(1):77-83.

10. Hossein-Zadeh NG, Ardalan M. Cow-specific risk factors for retained placenta, metritis and clinical mastitis in holstein cows. *Vet Res Commun*. 2011;35(6):345-354.

11. Opsomer G, De Kruif A. Metritis en endometritis bij hoogproductief melkvee. *Vlaam Diergeneeskd Tijdschr*. 2009;78(2):83-88.

12. van den Borne BHP, van Schaik G, Lam TJGM, Nielen M. Variation in herd level mastitis indicators between primi- and multiparae in dutch dairy herds. *Prev Vet Med*. 2010;96(1–2):49-55.

13. Barkema HW, Schukken YH, Lam TJGM, Beiboer ML, Benedictus G, Brand A. Management practices associated with the incidence rate of clinical mastitis. *J Dairy Sci*. 1999;82(8):1643-1654.

14. Huijps K, Lam TJGM, Hogeveen H. Costs of mastitis: Facts and perception. *J Diary Res*. 2008;75(1):113-120.

15. Hasala T, Huijps K, Østerås O, Hogeveen H. Economic effects of bovine mastitis and mastitis management: A review. *Veterinary Quarterly*. 2007;29(1):18-31.

16. Han Y-, Kim I-. Risk factors for retained placenta and the effect of retained placenta on the occurrence of postpartum diseases and subsequent reproductive performance in dairy cows. *Journal of Veterinary Science*. 2005;6(1):53-59.

17. LeBlanc SJ. Postpartum uterine disease and dairy herd reproductive performance: A review. *The Veterinary Journal*. 2008;176(1):102-114.

18. Kimura K, Goff JP, Kehrli Jr. ME, Reinhardt TA. Decreased neutrophil function as a cause of retained placenta in dairy cattle. *J Dairy Sci*. 2002;85(3):544-550.

19. Kossaibati MA, Esslemont RJ. The costs of production diseases in dairy herds in england. *The Veterinary Journal*. 1997;154(1):41-51.

20. Azawi OI. Postpartum uterine infection in cattle. *Anim Reprod Sci*. 2008;105(3-4):187-208.

21. Smart ME, Gudmundson J, Christensen DA. Trace mineral deficiencies in cattle: A review. *Can Vet J*. 1981;22(12):372-376.

22. Spears JW, Engle TW. Feed supplements: Microminerals. In: *Feed ingredients*. Elsevier; 2011:378-383.

23. Failla ML. Trace elements and host defense: Recent advances and continuing challenges. *J Nutr*. 2003;133(5 SUPPL. 2):1443S-1447S.

24. *Tabellenboek veevoeding 2010.* 8th ed. Den Haag: Productschap Diervoeder; 2010.

25. MacPerson A. Trace element deficiency in ruminants. *Outlook on Agriculture*. 1989;18(3):124-132.

26. Wintergerst ES, Maggini S, Hornig DH. Contribution of selected vitamins and trace elements to immune function. *Ann Nutr Metab*. 2007;51(4):301-323.

27. Suttle NF, Jones DG. Recent developments in trace element metabolism and function: Trace elements, disease resistance and immune responsiveness in ruminants. *J Nutr*. 1989;119(7):1055-1061.

28. Machado VS, Bicalho MLS, Pereira RV, et al. Effect of an injectable trace mineral supplement containing selenium, copper, zinc, and manganese on the health and production of lactating holstein cows. *Vet J*. 2013;197(2):451-456.

29. Cook JG, Green MJ. Reduced incidence of retained fetal membranes in dairy herds supplemented with iodine, selenium and cobalt. *Vet Rec*. 2007;161(18):625-626.

30. Arthur JR, McKenzie RC, Beckett GJ. Selenium in the immune system. *J Nutr*. 2003;133(5 SUPPL. 2):1457S-1459S.

31. Spears JW. Trace mineral bioavailability in ruminants. *J Nutr*. 2003;133(5 SUPPL. 2):1506S-1509S.

32. Nielsen C, Stengärde L, Bergsten C, Emanuelson U. Relationship between herd-level incidence rate of energy-related postpartum diseases, general risk factors and claw lesions in individual dairy cows recorded at maintenance claw trimming. *Acta Vet Scand*. 2013:55.

33. Drackley JK. ADSA foundation scholar award: Biology of dairy cows during the transition period: The final frontier? *J Dairy Sci*. 1999;82(11):2259-2273.

34. Scaletti RW, Trammell DS, Smith BA, Harmon RJ. Role of dietary copper in enhancing resistance to escherichia coli mastitis. *J Dairy Sci*. 2003;86(4):1240-1249.

35. Weiss WP, Hogan JS, Todhunter DA, Smith KL. Effect of vitamin E supplementation in diets with a low concentration of selenium on mammary gland health of dairy cows. *J Dairy Sci*. 1997;80(8):1728-1737.

36. Hemingway RG. The influences of dietary selenium and vitamin E intakes on milk somatic cell counts and mastitis in cows. *Vet Res Commun*. 1999;23(8):481-499.

37. Mulligan FJ, Doherty ML. Production diseases of the transition cow. *Veterinary Journal*. 2008;176(1):3-9.

38. Heinrichs AJ, Costello SS, Jones CM. Control of heifer mastitis by nutrition. *Vet Microbiol*. 2009;134(1-2):172-176.

Attachment 1: Distribution of health events among affected cows.

The following tables and figures show the distribution of mastitis, retentio secundinarum and endometritis in the herds used for this study in the years 2012 and 2013.

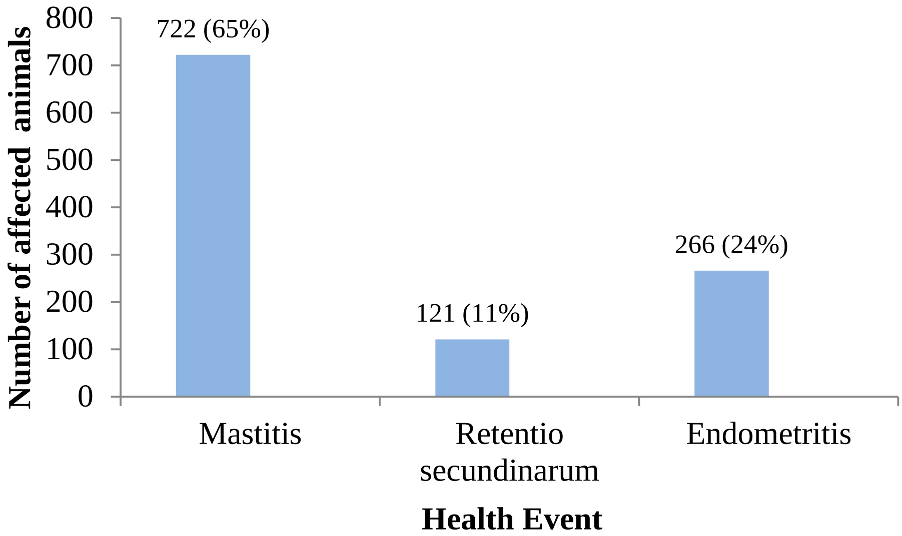
2012

Table 13**:** Number of health events which occurred in 2012 in the herds participating in the study.

Health event	N	%
Mastitis	722	65
Retentio secundinarum	121	11
Endometritis	266	24
Total health events	1109	100

The first column represents the health event, the second column the number of animals affected per health event, the third column the percentage of animals effected per health event. The total number of cows >22 months in 2012 was 1718.

Figure 5: Distribution of mastitis, retentio secundinarum and endometritis between affected cows in 2012.



Mastitis represents the most cases among the affected cows used in this study(65%). Endometritis represents 24% of the health events among the affected cows. Retentio secundinarum represents 11% of the health events among the cows used in this study. The total number of affected cows was 1109. Total number of cows used in this study in 2012 was 1718.

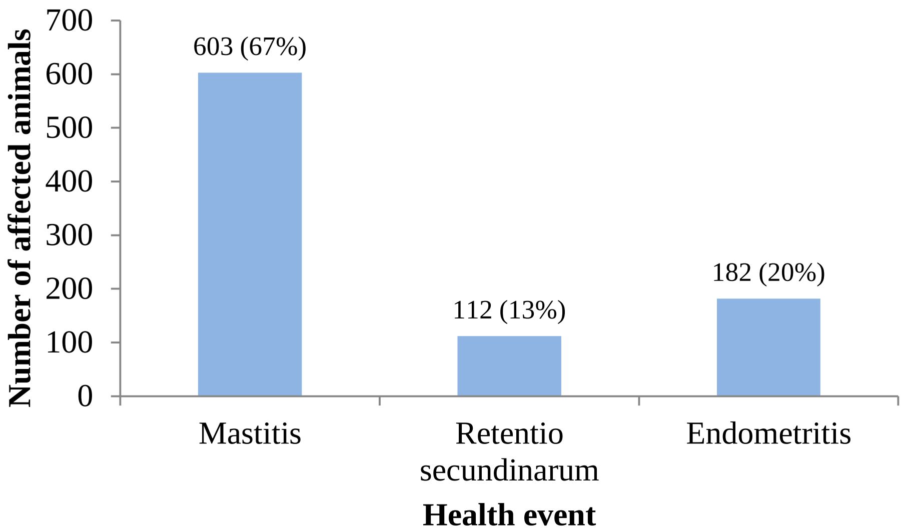
2013

Table 24**:** Number of health events which occurred in 2013 in the herds participating in the study.

Health event	N	%
Mastitis	603	67
Retentio secundinarum	112	13
Endometritis	182	20
Total health events	897	100

The first column represents the health event, the second column the number of animals affected per health event, the third column the percentage of animals effected per health event. The total number of cows >22 months in 2013 was 1913.

Figure 6: Distribution of mastitis, retentio secundinarum and endometritis between affected cows in 2012.



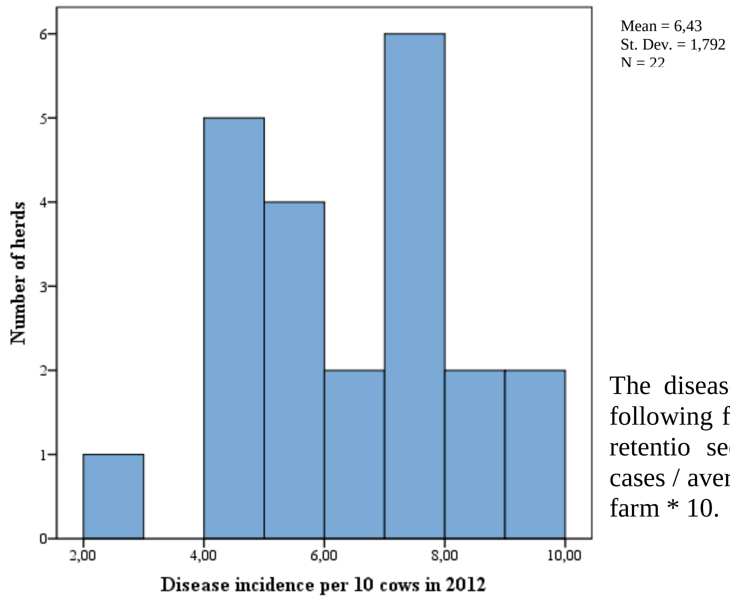
Mastitis represents the most cases among the affected cows used in this study(67%). Endometritis represents 20% of the health events among the affected cows. Retentio secundinarum represents 13% of the health events among the cows used in this study. The total number of affected cows was 897. Total number of cows used in this study in 2013 was 1913.

Attachment 2: Distribution of disease incidence

The following histograms show the distribution of the total disease incidence per 10 cows and the disease incidence per 10 cows of mastitis, retentio secundinarum and endometritis among the herds participating in this study during 2012 and 2013.

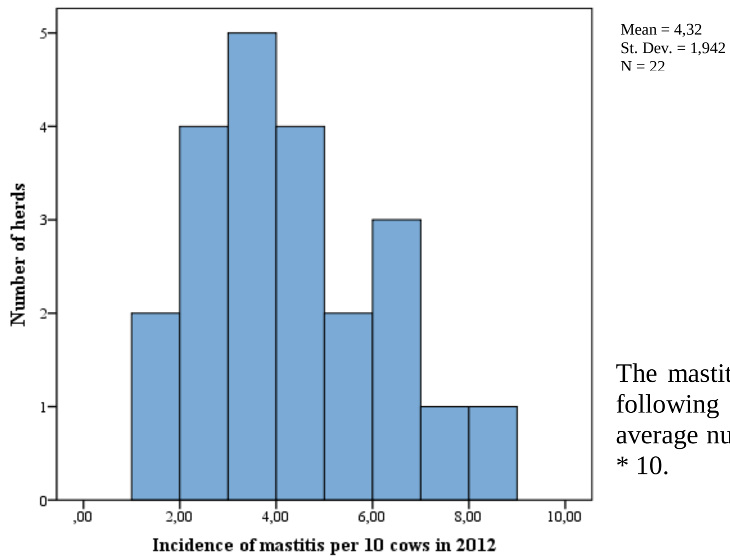
2012

Figure 7: Distribution of disease incidence per 10 cows in 2012 among the herds participating in the study.



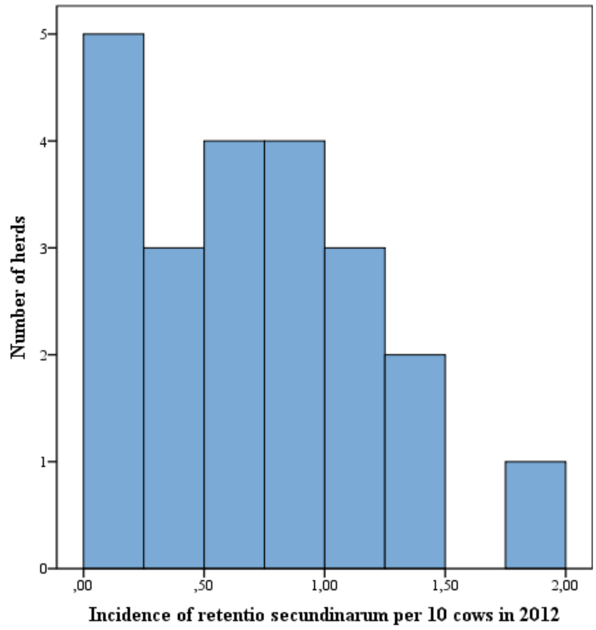
The disease incidence per herd was calculated with the following formula: Number of mastitis cases + number of retentio secundinarum cases + number of endometritis cases / average number of cows >22 months present on the farm * 10.

Figure 8: Distribution of mastitis incidence per 10 cows in 2012 among the herds participating in this study.



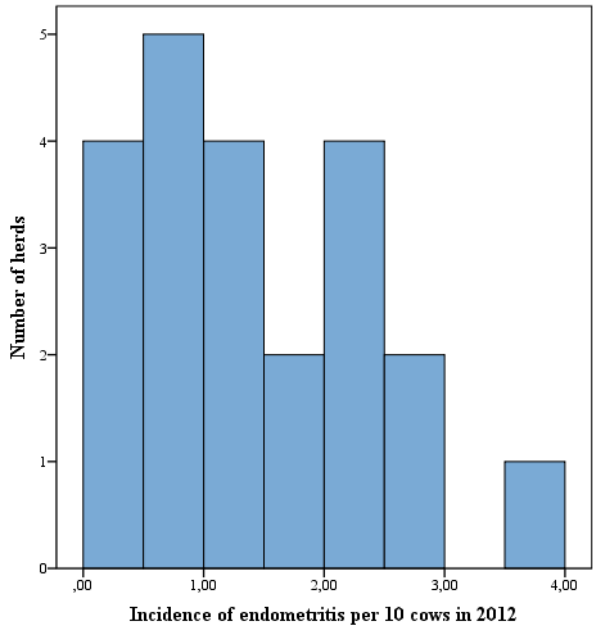
The mastitis incidence per herd was calculated with the following formula: Total number of mastitis cases / average number of cows >22 months present on the farm * 10.

Figure 9: Distribution of retentio secundinarum incidence per 10 cows in 2012 among the herds participating in this study



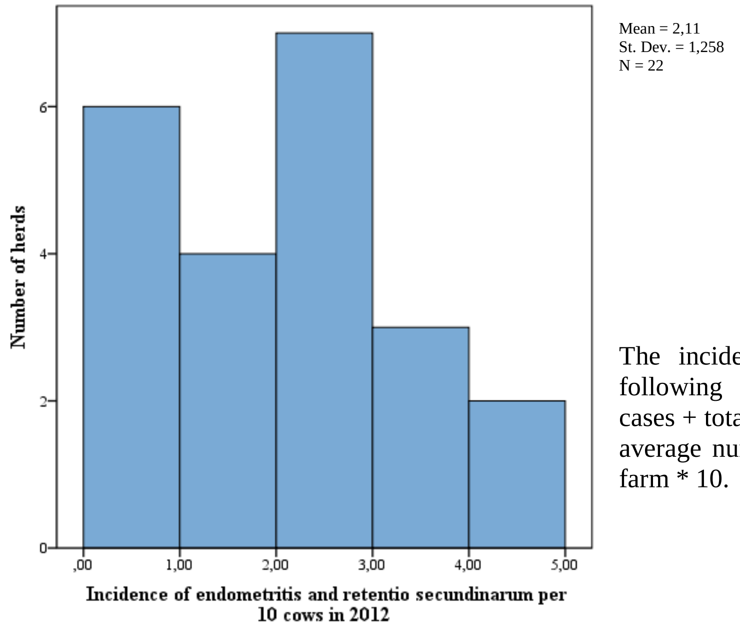
The retentio secundinarum incidence per herd was calculated with the following formula: Total number of retentio secundinarum cases / average number of cows >22 months present on the farm * 10.

Figure 10: Distribution of endometritis incidence per 10 cows in 2012 among the herds participating in this study.



The endometritis incidence per herd was calculated with the following formula: Total number of endometritis cases / average number of cows>22 months present on the farm * 10.

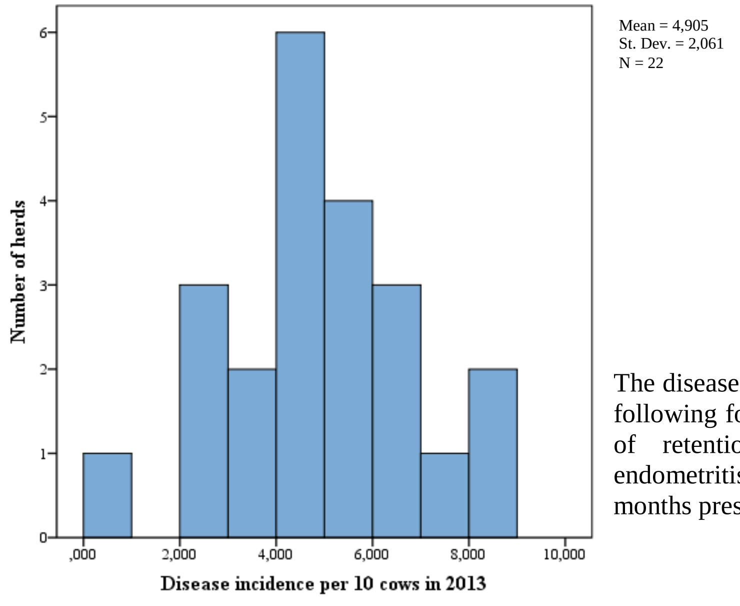
Figure 11: Distribution of endometritis and retentio secundinarum incidence per 10 cows in 2012 among the herds participating in this study.



The incidence per herd was calculated with the following formula: Total number of endometritis cases + total number of retentio secundinarum cases / average number of cows >22 months present on the farm * 10.

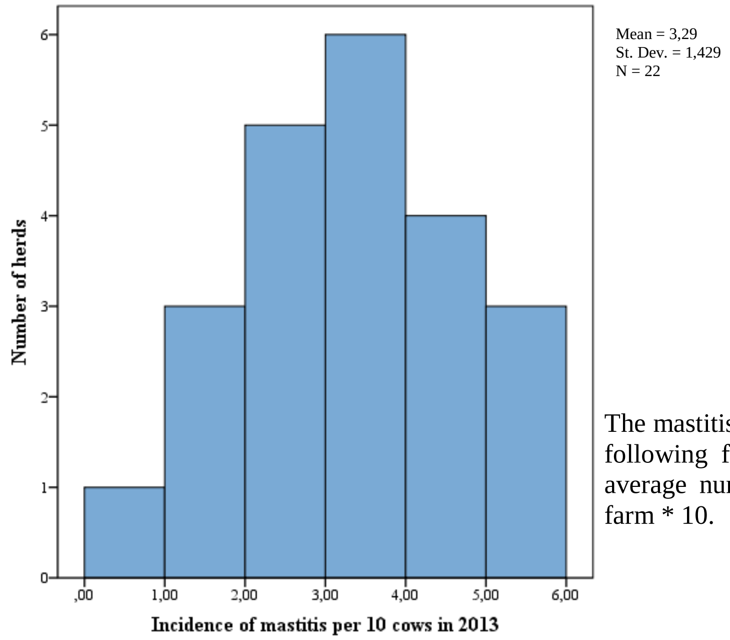
2013

Figure 12: Distribution of disease incidence per 10 cows in 2013 among the herds participating in the study.



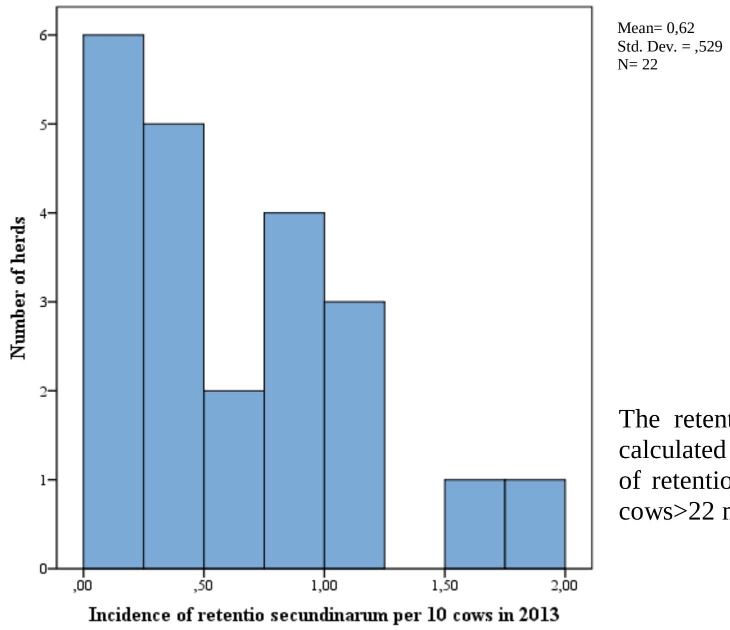
The disease incidence per herd was calculated with the following formula: number of mastitis cases + number of retentio secundinarum cases + number of endometritis cases / average number of cows >22 months present on the farm *10.

Figure 13: Distribution of mastitis incidence per 10 cows in 2013 among the herds participating in this study



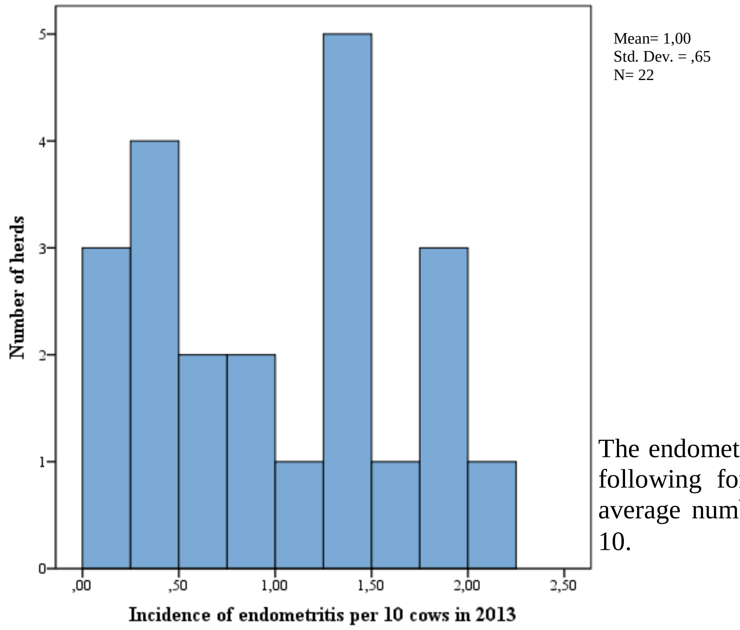
The mastitis incidence per herd was calculated with the following formula: Total number of mastitis cases / average number of cows >22 months present on the farm * 10.

Figure 14: Distribution of retentio secundinarum incidence per 10 cows in 2012 among the herds participating in this study.



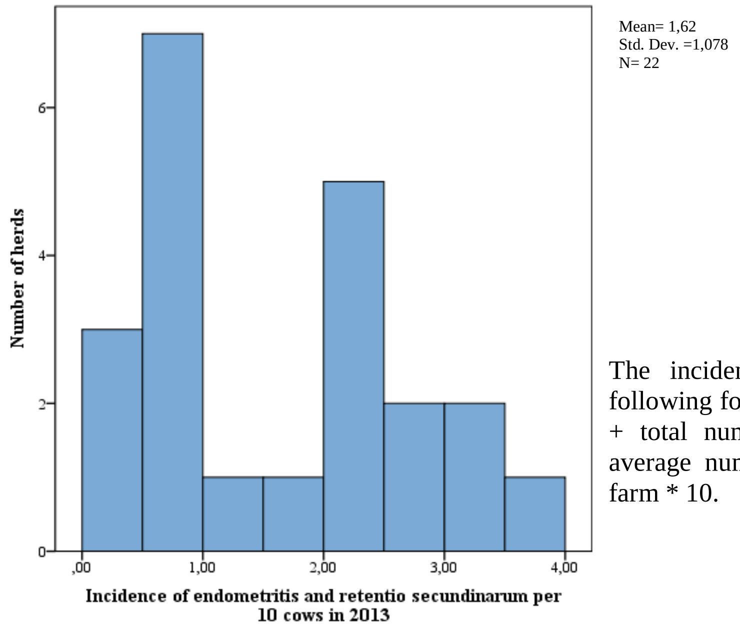
The retentio secundinarum incidence per herd was calculated with the following formula: Total number of retentio secundinarum cases / average number of cows>22 months present on the farm * 10.

Figure 16: Distribution of endometritis incidence per 10 cows in 2013 among the herds participating in this study.



The endometritis incidence per herd was calculated with the following formula: Total number of endometritis cases / average number of cows>22 months present on the farm * 10.

Figure 17: Distribution of endometritis and retentio secundinarum incidence per 10 cows in 2013 among the herds participating in this study.



The incidence per herd was calculated with the following formula: Total number of endometritis cases + total number of retentio secundinarum cases / average number of cows>22 months present on the farm * 10.

Attachment 3: Disease incidence categories

Table 25: Result of the calculation of the percentiles for the dependent variables disease incidence 2012 and 2013.

		Disease incidence per 10 cows in 2012	Disease incidence per 10 cows in 2013
Mean		6,428	4,905
Percentiles	25 (lower quartile)	4,798	3,261
	50	6,609	4,841
	75 (upper quartile)	7,657	6,529

The lower and upper quartile of disease incidence per 10 cows in 2012 and 2013 was used to categorize the herds in low and high incidence herds respectively. The disease incidence per 10 cows was calculated with the following formula: Number of mastitis cases + number of retentio secundinarum cases + number of endometritis cases / average number of cows>22 months present on the farm * 10.

Table 16: Result of the calculation of the percentiles for the dependent variables mastitis incidence in 2012 and 2013.

		Incidence of mastitis per 10 cows in 2012	Incidence of mastitis per 10 cows in 2013
Mean		4,315	3,289
Percentiles	25 (lower quartile)	2,710	2,089
	50	3,892	3,478
	75 (upper quartile)	5,851	4,275

Mastitis was responsible for the majority of the health events in the variable 'Disease incidence per 10 cows' in 2012 (65%) and 2013 (67%). To obtain more accurate results during analysis, this variable was split into two additional variables. The mastitis incidence per herd was calculated with the following formula: Total number of mastitis cases / average number of cows >22 months present on the farm * 10. The lower and upper quartile were used to categorize the herds in low and high mastitis incidence herds respectively.

Table 17: Result of the calculation of the percentiles for the dependent variables endometritis and retentio secundinarum incidence in 2012 and 2013.

		Incidence of retentio secundinarum and endometritis in 2012	Incidence of retentio secundinarum and endometritis in 2013
Mean		2,113	1,616
Percentiles	25 (lower quartile)	0,914	0,569
	50	2,483	1,404
	75 (upper quartile)	2,842	2,485

Retentio secundinarum and endometritis were responsible for the minority of the health events in the variable 'Disease incidence per 10 cows' in 2012 and 2013. The two variables 'Incidence of retentio secundinarum per 10 cows' and 'Incidence of endometritis per 10 cows' have been taken together as one variable, to obtain more accurate results during analysis. The lower and upper quartile were used to categorize the herds in low and high incidence herds respectively. The incidence per herd was calculated with the following formula: Total number of endometritis cases + total number of retentio secundinarum cases / average number of cows>22 months present on the farm * 10

2012

Figure 18: Bar chart showing the distribution of herds between the categories of disease incidence per 10 cows in 2012.

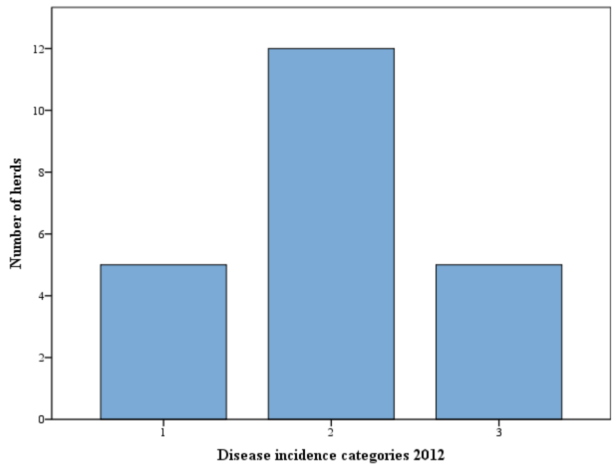


Figure 19: Bar chart showing the distribution of herds between the categories of mastitis incidence per 10 cows in 2012.

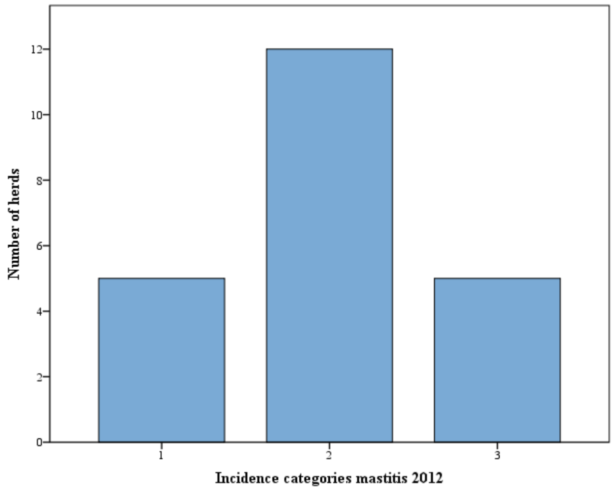
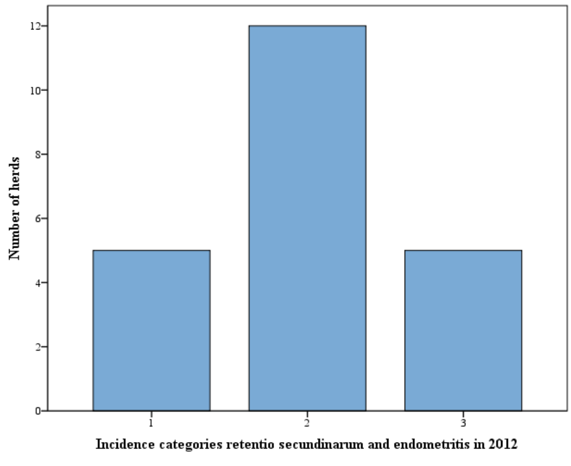


Figure 20: Bar chart showing the distribution of herds between the categories of retentio secundinarum and endometritis incidence per 10 cows in 2012.



2013

Figure 21: Bar chart showing the distribution of herds between the categories of disease incidence per 10 cows in 2013.

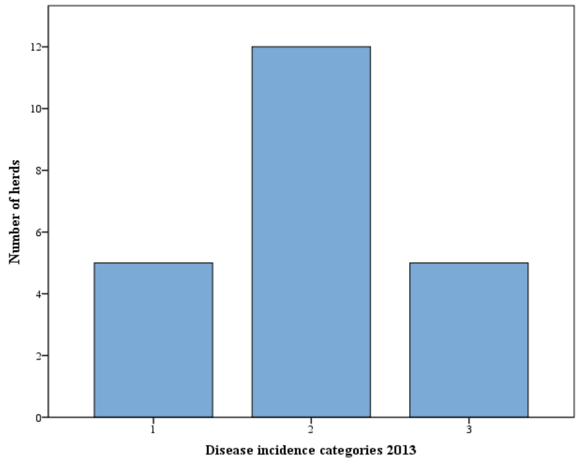


Figure 22: Bar chart showing the distribution of herds between the categories of mastitis incidence per 10 cows in 2013.

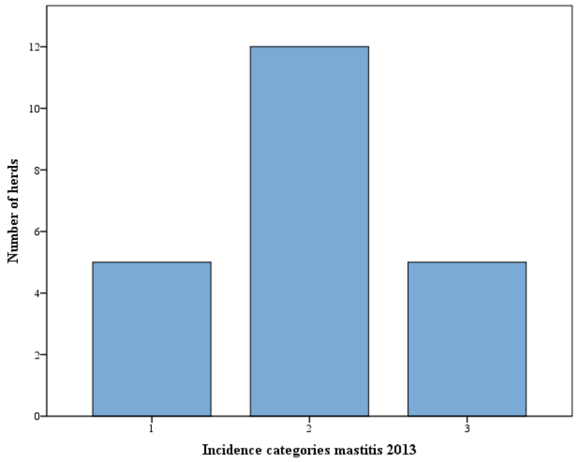
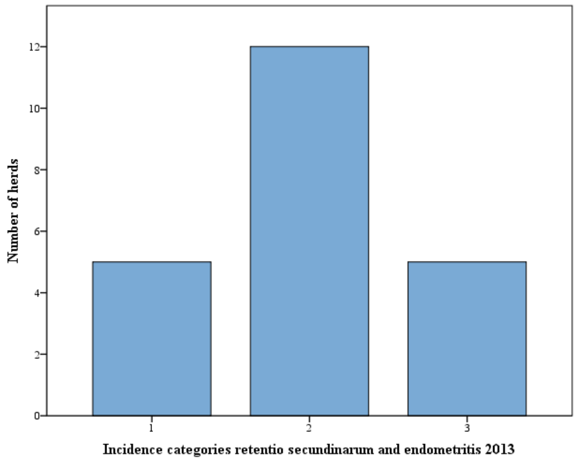


Figure 23: Bar chart showing the distribution of herds between the categories of retentio secundinarum and endometritis incidence per 10 cows in 2013.



Attachment 4: Results univariate regression analysis

The tables in this attachment show the results of the univariate regression analysis. The first column represents the independent variable, i.e. the trace element content in the diet separated in winter and summer period and lactation and dry period for cobalt, selenium and zinc. For copper the lactation period was separated in two periods; until 200 days into lactation and after 200 days into lactation. The regression coefficient is shown in the second column, the p-value in the third column. Normally distributed residuals, in column four, and homoscedasticity in column five are essential terms for linear regression analysis.

Table 38: Results of the univariate regression analysis with disease incidence per 10 cows in 2012 as dependent variable.

Independent variable	Regression coefficient	P-value	Distribution residuals	Scatterplot
Cobalt lactation winter period	0,000	0,820	Normal	Random
Cobalt dry winter period	0,001	0,555	Normal	Random
Cobalt lactation summer period	0,000	0,841	Normal	Random
Cobalt dry summer period	0,001	0,626	Normal	Random
Copper <200 days winter period	-0,002	0,761	Normal	Random
Copper >200 days winter period	-0,10	0,259	Normal	Random
Copper dry winter period	-0,005	0,409	Normal	Random
Copper <200 days summer period	0,010	0,165	Normal	Random
Copper >200 days summer period	0,010	0,247	Normal	Random
Copper dry summer period	-0,003	0,659	Normal	Random
Selenium lactation winter period	-0,007	0,429	Normal	Random
Selenium dry winter period	-0,003	0,363	Normal	Random
Selenium lactation summer period	0,001	0,881	Normal	Random
Selenium dry summer period	-0,005	0,214	Normal	Random
Zinc lactation winter period	-0,017	0,129	Normal	Random
Zinc dry winter period	-0,006	0,259	Normal	Random
Zinc lactation summer period	0,001	0,923	Normal	Random
Zinc dry summer period	-0,003	0,593	Normal	Random

Independent variables with p-value<0,20 are used for multivariate regression analysis. The multivariate regression analysis was performed with copper < 200 days during summer period and zinc lactation during winter period, with no significant (p<0,05) results.

Table 19: Results of the univariate regression analysis with disease incidence per 10 cows in 2013 as dependent variable.

Independent variable	Regression coefficient	P-value	Distribution residuals	Scatterplot
Cobalt lactation winter period	0,001	0,703	Normal	Random
Cobalt dry winter period	0,001	0,751	Normal	Random
Cobalt lactation summer period	0,002	0,253	Normal	Random
Cobalt dry summer period	3,978^-5	0,989	Normal	Random
Copper <200 days winter period	-0,010	0,212	Normal	Random
Copper >200 days winter period	-0,020	0,041	Normal	Random
Copper dry winter period	-0,003	0,662	Normal	Random
Copper <200 days summer period	0,000	0,954	Normal	Random
Copper >200 days summer period	-0,004	0,686	Normal	Random
Copper dry summer period	-0,002	0,776	Normal	Random
Selenium lactation winter period	-0,010	0,339	Normal	Random
Selenium dry winter period	-0,003	0,418	Normal	Random
Selenium lactation summer period	-0,001	0,888	Normal	Random
Selenium dry summer period	-0,005	0,254	Normal	Random
Zinc lactation winter period	-0,011	0,404	Normal	Random
Zinc dry winter period	-0,008	0,237	Normal	Random
Zinc lactation summer period	0,008	0,572	Normal	Random
Zinc dry summer period	-0,003	0,565	Normal	Random

Independent variables with p-value<0,20 are used for multivariate regression analysis. Copper > 200 days during the winter period is the only independent variable with p<0,20, therefor multivariate regression analysis could not be executed for this dependent variable.

Attachment 5: Trace element groups

Table 20: The eighty different trace element groups before regrouping.

	Season	Lactation stage	Days into lactation
Primiparous	Winter	Dry	
		Lactation	0-14 days
			15-100 days
			101-200 days
			>200 days
	Summer	Dry	
		Lactation	0-14 days
			15-100 days
			101-200 days
			>200 days
Multiparous	Winter	Dry	
		Lactation	0-14 days
			15-100 days
			101-200 days
			>200 days
	Summer	Dry	
		Lactation	0-14 days
			15-100 days
			101-200 days
			>200 days

Initially the trace element content in the diet was calculated for twenty different groups as showed above. The large amount of different groups made analysis of the data difficult, therefor regrouping of the variables was conducted based upon the categorization (1-4) of the trace element between the herds. If the trace element content in the diet of a particular group corresponded with the trace element content of another group, the groups were combined.

After regrouping the trace elements eighteen new independent variables were formed:

- Cobalt lactation winter period
- Cobalt dry winter period
- Cobalt lactation summer period
- Cobalt dry summer period

- Selenium lactation winter period
- Selenium dry winter period
- Selenium lactation summer period
- Selenium dry summer period

- Copper < 200 days winter period
- Copper > 200 days winter period
- Copper dry winter period
- Copper < 200 days summer period
- Copper > 200 days summer period
- Copper dry summer period

- Zinc lactation winter period
- Zinc dry winter period
- Zinc lactation summer period
- Zinc dry summer period