

## Monitoring different causal patterns of bovine abortion syndrome

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### Abstract

Bovine Abortion Syndrome (BAS) has a significant impact on commercial dairies. Abortion rates vary between 2 and 20 % and there is little information regarding non-infectious factors affecting fetal stages. We analyzed the joint relationship of infectious and non-infectious factors with the probability of abortion in dairy cows. We used dairy cows from the Ñuble province of Chile (n = 148) at 42 days of pregnancy. We then took monthly blood samples until abortion or calving. We determined plasma concentrations for the following: amino transferase, albumin, cholesterol, globulin, fibrinogen,  $\beta$ -hydroxybutyrate, retinol,  $\alpha$ -tocopherol, calcium, phosphorus, glutathione peroxidase, total proteins and urea. Serology was performed for infectious diseases and body conditions were recorded. Our study used a multivariable logistic regression model and proportional hazard regression. There was an ( $P < 0.05$ ) association with abortion, ( $OR = 4.27$ ) for *Neospora caninum* and ( $HR = 94.35$ ) for *Leptospira interrogans*, with a low cholesterol concentration ( $P < 0.05$ ) in the fourth month of pregnancy ( $OR = 0.61$ ), and a decrease in body condition from month three to four ( $OR = 0.32$ ). BAS is associated with negative energy balances, protective effects of high cholesterol plasma concentrations in the first trimester of gestation and incidences of *Neospora caninum*, *Leptospira interrogans*, and a conjunction of the two. It is important to provide epidemiological surveillance tools that help anticipate herd reproductive losses through management measures, infectious disease control and proper nutritional management.

**Keywords:** bovine abortion syndrome; energy balance; cholesterol; neosporosis; leptospirosis; dairy cows.

Submitted: 2018-09-20

Accepted: 2021-11-17

Published: 2022-06-28

Additional information and declarations  
can be found on page 17

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Cite this as:

Gädicke L'Huissier P, Chihuailaf R, Letelier R, Allende R, Ruiz A, Junod T. Monitoring different causal patterns of bovine abortion syndrome. Veterinaria México OA. 2022;9. doi: 10.22201/fmvz.24486760e.2022.652

## Study contribution

Bovine Abortion Syndrome is multicausal, can be of infectious or non-infectious origin, which makes it difficult to establish the cause in many cases. The analysis of the joint relationship of infectious and non-infectious factors with the probability of abortion in dairy cows using an integrated methodology is needed. If the traditional methodology had been followed in the 18 cases of abortions recorded in the followed cohort, it means, using only routine diagnostic tests for infectious diseases, the etiological agent would have been suspected in 6 of them. With the complementary methods applied in this work, we found that was associated with negative energy balances, protective effects of high cholesterol plasma concentrations in the first trimester of gestation and incidences of *Neospora caninum*, *Leptospira interrogans*, and a conjunction of them.

## Introduction

Bovine Abortion Syndrome (BAS) is multicausal. Diseases and pathologies that have been diagnosed by the Agricultural and Livestock Service (SAG) since 1999 showed that Bovine Viral Diarrhea Virus (BVD), *Neospora caninum*, *Brucella abortus* and *Leptospira* spp.<sup>(1, 2)</sup> are the main infectious causes of abortion. In dairy herds, this syndrome creates limitations for production given the economic losses it entails.<sup>(3, 4)</sup> Abortion can occur sporadically, endemically or in the form of an outbreak, and can be of infectious or non-infectious origin, which makes it difficult to establish the cause in many cases.<sup>(5)</sup>

Abortion is a significant factor in the loss of cows, with an impact of 30 to 40 % losses of total replacements, and an even greater impact when applied to animals with remaining useful life or animals of great genetic value within the herd.<sup>(4)</sup> Thus, the control and monitoring of infectious and non-infectious diseases which can trigger BAS in the herd is of vital importance; not doing so results in heavy consequences, such as the decreases in milk production, potential replacements and fertility, as well as increases in feeding costs, medical treatments, artificial inseminations and slaughter rates.<sup>(6, 7)</sup>

It has been noted that metabolic alterations associated to nutrition and energy required by the animals negatively affects reproductive herd performance, as is the case with high productions of milk and cystic ovaries.<sup>(8)</sup> Along the same line,<sup>(9)</sup> indicated that increases in individual milk production, regarding the previous lactation, increases the risk of abortion, placental retention, metritis and silent estrus. Additionally, a decrease in body condition can increase losses from 40 to 90 days of gestation, due to a decrease in progesterone production by the corpus luteum.<sup>(10)</sup>

The hypothesis proposed in this paper is that metabolic imbalances of energy and/or protein are important non-infectious causes of BAS. The objective of our study was to detect and describe cases of abortion in dairy cows in relation to the metabolic imbalances and infectious diseases that cause abortion in the area.

The contribution of this study is the joint monitoring of possible infectious and non-infectious factors that may be involved in BAS, allowing the analysis of their interactions, which may constitute different causal patterns.

## Materials and methods

### *Ethical statement*

The Bioethical Committee of Veterinary Faculty from Universidad de Concepción approved the research (CE-17-3-2011).

### *Study design*

A prospective observational cohort study was developed to analyze possible relationships between bovine abortion syndrome, metabolic imbalances and infectious diseases prevailing. Cows were monitored and sampled monthly during pregnancy, completing seven samplings until the dry period (between month two and eight of gestation). Pregnancy was reviewed monthly by rectal palpation and/or ultrasound (Honda Electronics® Ltd. hs-1500 ultrasound scanner with convex multi-frequency transducer). The cows used in the sample have had more than one calving (average 2.3 calves) and were on average 4.2 years of age. The ethical committee of the relevant department approved the study.

### *Sample size*

To detect and describe cases of abortion in dairy cows, a minimum of 137 animals were calculated to be needed, based on an estimate that considers a confidence level of 95 %, population size of 900 and 18 possible cases to detect. This implies an expected minimum prevalence of 0.02 % and a sample fraction of 15.2 %, using the Win Episcopo 2.0 program.<sup>(11)</sup> The two properties in the study were designated as premises a and b, selected for convenience according to owner consent, with abortion rates in previous years of ( $\geq 8\%$ ) and reliable reproductive parameters.

### *Animal selection, characteristics, and locations*

The two dairies evaluated belong to the Bulnes commune of the Ñuble province, Biobío region in Chile. The dairy cows were of the Holstein Friesian breed, with 750 and 500 cows respectively on sites A and B. The minimum sample size was 137 cows; however, due to the possible loss of cows during follow-ups, 148 cows were monitored proportionally to the total size of each herd (90 from farm A and 58 from farm B). Holstein-Friesian breed animals were included in the study after at least 42 days of gestation, calculated from the date of their last artificial insemination until their entrance to the dry period or abortion.

### *Blood sampling*

Blood samples were taken from each cow by veno-coccygeal puncture, using Venoject® blood tubes, two tubes without anticoagulant and one with anticoagulant (5 mL of blood in each tube). Blood samples were identified by cow and farm. Within the same day, samples were transported in a portable refrigerator at 5°C to the "Microbiology Laboratory" of the "Veterinary Sciences Faculty", Universidad de Concepción-Chillán, campus in Chile. Samples were centrifuged at 1 800 rpm for ten minutes in order to separate the serum from the clot, obtaining serum aliquots that were frozen at -18°C. In the event that there was not a complete separation be-

tween the serum and the clot, samples were centrifuged at 2 500 rpm for five minutes. Dynamics of antibodies for prevailing diseases in the area were analyzed.<sup>(12)</sup> Evidence of seroconversion was sought for bovine viral diarrhoea, leptospirosis and neosporosis, which was compatible with the period in which abortions occurred.

### Conditions evaluated in each sampling and methodology used:

#### Body condition evaluation

The five-point scale indicated by Edmonson et al.<sup>(13)</sup> was used, considering the degree of subcutaneous fat: condition 1 being emaciated and bony cows, while condition five would show a greater evidence of fatty deposit.

#### Metabolic profile analysis

Blood serum samples were sent to the "Clinical Laboratory" of the "Faculty of Natural Resources", "School of Veterinary Medicine", Universidad Católica de Temuco, Chile, and analyzed to determine plasma concentrations of metabolites by means of the following methodologies:

- Aspartate aminotransferase (AST, U/L): colorimetric kinetic method using a commercial reagent (got AST®, Wiener Lab) in a spectrophotometer.
- Albumin (g/L): bromocresol green colorimetric method, measured in an autoanalyzer at 550 nm.
- Cholesterol (mmol/L): colorimetric kinetic method using a commercial reagent (Cholesterol®, Human) in spectrophotometer Metrolab 2 300 plus.
- Globulin (g/L): calculation of the difference between plasma concentrations of total protein and albumin.
- $\beta$ -hydroxybutyrate (mmol/L): 3-betahydroxybutyrate dehydrogenase-dependent enzyme method using a commercial reagent, measured at 340 nm.
- $\alpha$ -tocopherol and retinol ( $\mu\text{g/mL}$  of plasma): reverse phase high performance liquid chromatography (HPLC) in a Perkin Elmer chromatograph coupled to a uv detector, after lipid extraction, according to the methods indicated by Chihuilaf et al.<sup>(14)</sup> A Kromasil c18 column of  $150 \times 4.6 \text{ mm} \times 5 \mu\text{m}$  and 100 % methanol was used as a mobile phase at a flow rate of 1.5 mL/min for the first four minutes and 2 mL/min for ten minutes.
- Calcium (mmol/L): using o-cresolphthalein compleximetric colorimetric method, with a commercial reagent. Determination was carried out at 578 nm at a temperature of 37°C.
- Inorganic phosphate (mmol/L): molybdate/vanadate reaction method at 340 nm, at 37°C.
- Selenium ( $\mu\text{g}$  of hemoglobin): measurement of glutathione peroxidase (GPx) enzyme blood activity, through the NADPH-dependent kinetic technique using a commercial reagent at 340 nm.
- Total protein (g/L): through the Biuret method in a 550 nm auto-analyzer;
- Albumin: bromocresol green colorimetric method, measured in an autoanalyzer at 550 nm.
- Urea (mmol/L): through the GLDH kinetic enzymatic method, measured in an autoanalyzer at 340 nm.
- Serological analysis of infectious diseases: the IDEXX DVB Total Ab® (Bovine Viral Diarrhoea Virus (BVD) Antibody Test Kit) was used to diagnose BVD; the

IDEXX Neospora x2® test (*Neospora caninum* Antibody Test Kit) was used for neosporosis, by means of manufacturer recommended protocols (IDEXX and test with confidence of IDEXX Laboratories, Inc.), and for reading the scanner model Multiskan ex (© 2010 Thermo Fisher Scientific Inc.). With the optical densities obtained, we performed a weighted calculation according to manufacturer recommendations, to obtain cut-off limits. The MAT (Microscopic Agglutination Test) test was used to diagnose leptospirosis (*Leptospira interrogans*) for eight Serovars of *Leptospira interrogans*: *Grippityphosa* (Gr), *Canicola* (c), *Tarassovi* (t), *Hardjo* (h), *Pomona* (p), *Ballum* (b), *Autumnalis* (a), and *Copenhageni* (co), according to Cerqueira and Pircadeau.<sup>(15)</sup> The Rosa de Bengala diagnostic test was used to analyze the brucellosis disease, according to technical instructions for Rosa de Bengala analysis of "Agricultural and Livestock Services".

### Health analysis of each cow

The Wittwer<sup>(16)</sup> reference table for biochemical values of bovines was used to compare and classify the reference intervals (RI) of metabolites analyzed in cow biochemical profiles. According to the biochemical indicators found outside the herd reference intervals or normality, results were classified into different types of imbalances:<sup>(17)</sup>

- energy imbalance: low plasma cholesterol, high beta-hydroxybutyrate concentrations, as well as decreases in body conditions.
- protein imbalance: high plasma concentrations of urea, low total protein, albumin or globulin and high fibrinogen.
- unbalance of mineral metabolism: plasma concentrations outside reference ranges for calcium, phosphorus and selenium.
- damage to hepatocellular integrity: high plasma concentrations of AST and FA.
- metabolism of fat-soluble vitamins: plasma concentrations of retinol and alpha-tocopherol, outside the interquartile range indicated for cows in the dry period.
- background of infectious diseases: evidence of seroconversion of abortigenic type prevalent in the area. Seroconversion was sought during pregnancy for DVB, *Neospora caninum*, *Leptospira* sp. Brucellosis was controlled, as part of the sag national eradication program.

### Statistical analysis

Descriptive statistics were calculated for each sampling month of biochemical variables, body condition variations and infectious disease antibodies, for aborted cows and those who maintained pregnancy until the dry period.

The frequency of each metabolite over or under the RI, according to the imbalance indicator, was compared by month of pregnancy between aborted and non-aborted cows, homogeneity of frequencies was tested with Chi<sup>2</sup> or Fisher exact test with  $p < 0.05$ . The Pearson correlation between the fat-soluble vitamin's concentration and the antibodies titers for BVD and NEO was evaluated with  $p < 0.05$ .

A multivariate risk factor analysis for abortion in each sampling month was performed. The dependent variable for logistic regression was the condition of having

aborted or not. For the Cox's survival regression model, the dependent variable was the time of abortion and the final gestation condition. For both models, the independent variables were the metabolic profile indicators and the optical densities or titles of serological analysis. The condition of the linear effect in the log odds were post hoc evaluated. The identification of each cow was considered the within factor for repeated measure design. The aborted cows were contrasted with non-aborted cows in similar gestation month. When the cow aborted, it was no longer considered for the following time.

The logistic regression model and Cox's survival regression model were performed to evaluate risk factors and the time analysis. This was associated with pregnancy loss and infectious and metabolic parameters, considering repeated measures of the same cow until she aborted or not. The regressions were made with a significance level of 95 %, and confounders and two-level interactions were evaluated.<sup>(18)</sup> Analyses were performed using *Stata 11* software.<sup>(19)</sup>

First, a univariate logistic regression analysis was performed, identifying the variables with OR  $P < 0.2$ . The multivariate analysis was then carried forward stepwise to elaborate a prediction model to identify risk factors associated with abortion. A multivariate logistic regression and survival analysis was performed using Cox regression in a "step-by-step" manner (forward stepwise) to search for risk factors for abortion according to time of pregnancy. Confounding factors and interactions between variables were evaluated using logistic regression and survival models. The effect of each farm was controlled by including them in the models, with a 95 % significance level.<sup>(18)</sup> Data management and statistical analysis was developed using the *Stata 9 SE* program.<sup>(19)</sup>

## Results

Animal and herd characteristics. A total of 130 cows finished gestation: 82 from farm A (91 % fetal survival) and 48 from farm B (82.8 % fetal survival). There were 18 abortion cases in total, corresponding to a cumulative incidence rate (ci) of 12.2 %. Of the total number of abortions, eight occurred in farm A, which corresponds to a ci of 8.9 %, while farm B presented ten abortion cases, corresponding to a ci of 17.2 %. The abortions were concentrated in months 3-4 and 7-8 of the pregnancy periods. Proportions were always higher in farm B. A ci of 3.4 % was reported in month three, 5 % in month four, and 2.3 % in months seven and eight, of the total abortion cases, as shown in [Table 1](#).

### Energetic metabolism

- Cholesterol (Chol). Aborted cows presented plasma cholesterol concentrations under the ri at months three (40 %), and four (14.3 %) of gestation ([Table 4](#)). The normal cows had values under the ri at months three (6.3 %), and four (4.0 %) of gestation.  $P > 0.05$  was recorded for each month.
- $\beta$ -hydroxybutyrate (BHB). Aborted cows presented BHB concentrations over the ri in months three (40 %), four (14.3 %), seven (66.6 %), and eight (33.3 %) ([Table 4](#)). The normal cows had values over the ri in months three (36.7 %),

**Table 1.** Cumulative incidence rate of abortions, and the number of cows that aborted versus the total number of pregnant cows, according to farm and gestation month (% abortions/cows in gestation)

Gestation months	Farm		Total
	A	B	
2	0 (0/90)	0 (0/58)	0 (0/148)
3	5.6 (5/90)	0 (0/58)	3.4 (5/148)
4	0 (0/85)	12.7(7/55)	5 (7/140)
5	0 (0/85)	0 (0/48)	0 (0/133)
6	0 (0/83)	0 (0/48)	0 (0/133)
7	2.4 (2/83)	2.2 (1/46)	2.3 (3/129)
8	1.2(1/81)	4.4 (2/45)	2.3(3/126)
Total	8.9 (8/90)	17.2 (10/58)	12.2(18/148)

four (35.2 %), seven (29.5 %), and eight (21.7 %).  $P > 0.05$  was recorded for each month.

- Corporal condition. The cows that aborted had a higher percentage of animals that decreased in body condition by more than 0.5 points (22 %) from months three to four, with respect to the non-aborted group (17 %) ( $P < 0.05$ ).

### Protein metabolism

- Urea (u). Aborted cows presented u concentrations over the RI in month three (20 %) (Table 4). Fifty percent of the normal cows had values over the RI at the same time.  $P > 0.05$  was recorded for each month.
- Total protein (Pt). Aborted cows presented Pt concentrations under the RI in months three (40 %), four (14.3 %), and seven (16.6 %) (Table 4). The normal cows had values under the RI in months two (22.2 %), three (7.7 %), and seven (33.3 %).  $P < 0.05$  was recorded for month three and seven.
- Albumin (Alb). Aborted cows presented Alb concentrations over the RI in months three (20 %), seven (33.3 %), and eight (33.3 %) (Table 4). The normal cows had values over the RI in months three (11.1 %), seven (16.7 %), and eight (0 %) months.  $P > 0.05$  was recorded for each month.
- Globulins (Glob). Aborted cows presented Glob concentrations over the RI in month three (20 %) (Table 4). The normal cows had values over the RI in month three (8.3 %).  $P > 0.05$  was recorded for each month.
- Fibrinogen (Fib). Aborted cows presented Fib concentrations over the RI in months three (60 %), four (71.4 %), and seven (33.3 %) (Table 4). The normal cows had values over RI in months three (40 %), four (57.1), and seven (23. 1%).  $P > 0.05$  was recorded for each month.

### Mineral metabolism

- Calcium (ca). Ca plasma concentrations were within the RI throughout gestation for aborted and non-aborted cows ( $P > 0.05$ ).
- Phosphorus (P) and glutathione peroxidase (GPX). Aborted and non-aborted cows' phosphorus plasma concentrations and GPX enzymatic activity were recorded within the RI for aborted and non-aborted cows ( $P > 0.05$ ).

### Hepatocellular integrity

- Aspartate amino transferase (AST). Aborted cows presented AST concentrations over the RI in months three (20 %), seven (33.3 %), and eight (33.3 %) (Table 4). Normal cows had values over the RI in months three (13.5 %), seven (17.8 %), and eight (22.9 %).  $P > 0.05$  was recorded for each month.

### Liposoluble vitamin metabolism

- Retinol (Ret) and  $\alpha$ -Tocopherol (Toc). All cows during gestation had retinol values over the RI with no significant differences ( $P > 0.05$ ) found per gestation month between aborted and non-aborted cows.

### Infectious factors

- Bovine Viral Diarrhea Virus. The aborted and non-aborted cows tested positive for the BVD ELISA antibody test, which can be explained by the permanent vaccinations in both dairies. Also, seroconversion titres were not observed in successive samples (greater than 4 $\times$ ). In addition, all aborted cows tested negative for the BVD ELISA antigen test. However, throughout all pregnancies, the Q3 of aborted cows group was greater than that of non-aborted cows. The evidence is not sufficient to link any of the abortions directly with BVD. Median titers for aborted and non-aborted cows were  $P > 0.05$ .
- *Neosporora caninum* (neosporosis). Evidence of antibody seroconversion before the abortions were founded in months four (8.3 %), seven (66.6 %), and eight (33.3 %) (Table 4). The normal cows had seroconversion in months four (23.9 %), seven (19.4 %), and eight (11.9 %). This indicates the presence of the disease, but not all cases of seroconversion were associated with abortion.  $P < 0.05$  was recorded for proportions of positive cows in aborted and non-aborted cows.
- *Leptospira interrogans* 8 serovar diagnosis, and their titration by the MAT test, was performed. Serovars were the following: *L. interrogans* Serovar *Grippityphosa* (Gr), *L. int.* Serovar *Canicola* (Ca), *L. int.* Serovar *Tarassovi* (T), *L. int.* Serovar *Hardjo* (Ha), *L. int.* Serovar *Pomona* (Po), *L. int.* Serovar *Ballum* (Ba), *L. int.* Serovar *Autumnalis* (Au) and *L. int.* Serovar *Copenhageni* (Co). A greater proportion of reactive cows before abortion were found, corresponding to Serovars *Hardjo*, and *Grippityphosa*. According to the serology, two (11.1 %) of the total abortions can be linked to this disease. 4.9 % of the reactive cows were found in the normal cows, and  $P > 0.05$  was recorded for proportions of positive cows in aborted and non-aborted cows.
- *Brucella abortus*. All samples analyzed through the Rose Bengal test were negative in both farms, which was a response to the official eradication plan of the "Livestock Agricultural Service" in the region.<sup>(2)</sup>

### Multivariate analysis

The variables that were considered when elaborating the logistic regression model were those that had a significant difference ( $P < 0.2$ ) in the univariate analysis, prioritizing the two previous months and the month in which most abortions occurred.



**Table 2.** Cow abortion logistic regression results for infectious and non-infectious variables

Variable	OR	[ IC 95 % ]		P-value
Chol4	0.61	0.4	0.93	0.021
Var CC3-4	0.32	0.1	0.99	0.048
Neo4	0.18	0.04	0.89	0.0358
Neo5	4.27	1.36	13.44	0.0131

**Table 3.** Results for the Cox survival-regression model of infectious and noninfectious variables for cow abortion

Variable	Risk ratio	[ IC 95 % ]		P > z
Farm	0.00	3.98E-07	2.17	0.078
Chol4	0.21	0.06	0.74	0.015
Prod4	1.21	0.95	1.55	0.116
Neo5	25.44	1.30	496.63	0.033
Lepto6	94.35	4.00	2,222.32	0.005

Cholesterol plasma concentrations in month four had a protective effect for abortion (OR = 0.61), as did the increase in body condition in month three (OR = 0.32). In month five, this variable was a risk factor since there was a four times higher probability of abortion (OR = 4.27) (Table 2). The antibody titers for *Neospora caninum* in month four (OR = 0.18) has a confounder effect. Cholesterol in month four was a protective factor for the time of occurrence of abortion according to the Cox survival regression model, but higher titers were recorded for neosporosis in month five and leptospirosis in month six, which were abortion risk factors (Table 3).

### Probable causal patterns of the BAS in the analyzed farms.

Different possible causes were evidenced according to the results found for the analyzed farms (Table 4). Non-infectious factors predominate in farm A (energy imbalances, protein hepatocellular integrity, inflammatory processes), except in the case of an abortion identified due to neosporosis seropositivity. Infectious factors related to leptospirosis (Serovars Gr and Ha) and neosporosis predominated in farm B. However, negative energy balances and cholesterol protection factors were recorded in both farms as a predictor of gestation in month four. This metabolite, along with a BCS evaluation, is useful for evaluating energy imbalances in dairy herds. The correlation between retinol plasma and BVD titers was the median and was negative ( $r = -0.42$ ,  $P < 0.05$ ).

Regarding the factors related to the moment of abortion, metabolic profiles in the first third of gestation indicated energy imbalances (Chol↓, BNB↑), protein (Pt↓, Glob↑, Urea↑), hepatocellular damage (AST↑) and non-specific inflammatory processes (Fib↑); in the second third there were energy (Chol↓, BNB↑) and protein (Pt↓) imbalances, non-specific inflammatory processes (Fib↑) and prevalent infectious diseases such as *Neospora caninum* and *Leptospira interrogans* (Serovars *Grippotyphosa* and *Harjo*); in the last third there were energy (BNB↑) and protein (Pt↓, Alb↑) imbalances, hepatocellular damage (AST↑), non-specific inflammatory processes (Fib↑) and neosporosis.

The joint presentation of energy imbalances and leptospirosis or neosporosis is noteworthy. These factors could act as primary or secondary determining factors in abortion development, which should be analyzed further in future clinical studies.

## Discussion

The cumulative incidence rate of abortion in the study was 12.2 %, a value similar to that estimated by Gädicke et al.<sup>(20)</sup> in southern Chile. The highest cumulative incidence rates were in months three and four of pregnancy (3.4 % and 5 % respectively) according to Table 1. This is consistent with Gädicke and Monti,<sup>(4)</sup> who indicate that abortions occur more often during the first trimester than during other periods. Different values were found in a similar study, in which the probabilities of abortion during the first trimester were higher, with fewer probabilities in the second trimester than the other trimesters in conjunction: 44.1 %, 0.9 %, and 18.9 %, respectively.<sup>(21)</sup>

Different causes were evidenced in both farms studied. Non-infectious factors predominated in farm A (energy imbalances, protein hepatocellular integrity, inflammatory processes), except in the case of a neosporosis seropositivity abortion. Infectious factors related to leptospirosis and neosporosis predominated in farm B. However, in both farms, negative energy balances and cholesterol protection factors were recorded as predictors of gestation in month four. These predictors and BCS evaluations would be useful and economical tools for evaluating energy imbalances in dairy herds.<sup>(22)</sup>

When analyzing biochemical profiles throughout pregnancy, it was observed that the main metabolic imbalances at the herd level in the dairies analyzed were related to energy metabolism, liver integrity and protein metabolism. Aborted cows presented greater alterations in the form of negative energy balances and liver integrity affected by fat mobilization. Aborted cows also presented a more marked lack of synchrony between energy and degradable proteins in the rumen.

Cholesterol plasma concentrations were lower in sample four of aborted cows than non-aborted cows. This was associated with a protective effect (OR = 0.61,  $P < 0.05$ ) for the probability of abortion, reflecting those cows with higher cholesterol levels (always within the RI) would be less likely to abort. This metabolite can be considered as a nutritional indicator in cattle.<sup>(23)</sup> From a physiological point of view, this may reflect that certain cows adapt differently to productive demand changes and that some are more susceptible and cannot handle metabolic imbalances. Cholesterol plasma concentrations depend on diets, and especially on the type and amount of fat consumed. They increase when intakes are rich in fats, especially unsaturated fats, and decrease in states of malnutrition.<sup>(23)</sup> The dairies in which the study was conducted were advised by the same nutritionist, and the diets were formulated according to the lactation stages.

According to Duffield et al.,<sup>(24)</sup> the ability of cows to manage energy intake and demand in critical production periods is the most important aspect in maintaining healthy and productive lactation. A poor response to energy requirements can lead to a multitude of problems, including clinical illnesses and low milk production. It also puts cows at a higher risk of developing metabolic disorders.<sup>(25-28)</sup> An indicator of this poor adaptive response is an increase in ketone body concentrations

**Table 4.** Detailed analysis of each abortion case according to causal patterns and properties

Nº abortion	Month	Chol	BHB	Pt	Alb	Glob	Fib	Urea	AST	Neo	Lepto	Possible causal pattern of abortion
1	3	↓	↑				↑					Energy imbalance, inflammation
2	3		↑			↑						Energy imbalance, inflammation
3	3	↓		↓			↑	↑				Protein imbalance, inflammation
4	3				↑				↑			Hepatocellular damage, inflammation
5	3			↓			↑					Inflammation
6	4	↓	↑								Gr/200	Energy imbalance, leptospirosis
7	4										Ha/200	Leptospirosis
8	4						↑			+		Inflammation, neosporosis
9	4			↓			↑					Inflammation
10	4						↑					Inflammation
11	4						↑					Inflammation
12	4						↑					Inflammation
13	7		↑						↑	+		Energy imbalance, hepatocellular damage, neosporosis
14	7			↓	↑					+		Inflammation, neosporosis
15	7		↑				↑					Energy imbalance, Inflammation
16	8									+		Neosporosis
17	8		↑		↑							Energy imbalance, inflammation
18	8								↑			Hepatocellular damage

↓ = Plasmatic concentration under the RI for the species, ↑ = Plasmatic concentration over the RI for the species, +: neosporosis positive serology, Gr: *L. interrogans* Serovar, *Grippotyphosa*, Ha: *L. int.* Serovar *Hardjo*.

or ketonemia.<sup>(27)</sup> Within these metabolites is  $\beta$ -hydroxybutyrate, whose plasma concentrations at month two in aborted and non-aborted cows were within the RI, indicating a process of adipose tissue mobilization due to a significant energy imbalance at the herd level.<sup>(29)</sup> Additionally, throughout the course of the study in both farms, it was possible to register displacement of abomasum cases, a frequent metabolic disorder in dairy cows.<sup>(30, 31)</sup>

Blood urea interpretations in ruminants depend on protein and energy consumption, demonstrating that certain organisms do not have a strong mechanism of homeostasis to maintaining constant levels of urea in the blood, making it difficult to obtain normal reference levels.<sup>(32)</sup> In this study, pregnant cow plasma urea concentrations remained close to the upper reference interval. Only aborted cows in farm B presented medians notoriously above the RI. This was possibly due to increased protein consumption, or to diets with easily digestible protein or high levels of non-protein nitrogen, where there was greater absorption of the ammonia rumen, and therefore greater urea synthesis in the liver.

In the study conducted by Piccione et al.,<sup>(33)</sup> urea concentrations showed a statistically significant increase in the final pregnancy stage, compared to the other stages and at the beginning of lactation and compared to the remainder of lactation. It has been reported in a study on smaller ruminants that during the lactation period, urea concentrations increase due to higher energy requirements; at the end of pregnancy, concentrations clearly depend on protein intake.<sup>(34)</sup>

In addition, no relationship was found between the probability of abortion and urea plasma concentrations ( $P > 0.05$ ), nor with the time in which these abortions occurred, according to logistic regression results. However, authors indicate that high urea plasma concentrations affect the uterine environment. This can cause a toxic metabolic effect, leading to embryonic death,<sup>(35, 36)</sup> which can be a significant risk factor in early gestation stages. In farm B, significant differences were observed ( $P < 0.05$ ) in urea plasma concentrations in the first third of gestation when comparing aborted and non-aborted cows, which shows that herds go through negative energy balances that affect animal health.<sup>(37)</sup> However, negative energy balances do not affect energy, protein, mineral and hepatic metabolisms of some animals.<sup>(38)</sup> This is because some animals do not tolerate physiological and nutritional changes, since they are regulated by homeostasis.<sup>(39)</sup> According to some authors, certain blood metabolites such as non-esterified fatty acids (NEFA) are useful in measuring energy balances in ruminants, since they indicate fat mobilization and are more sensitive indicators of negative energy balances.<sup>(40-42)</sup>

Negative energy balances can also be reflected in negative variations of body condition. A higher percentage ( $P < 0.05$ ) of aborted cows had lower body conditions (more than 0.5 points on the BCS scale), compared to the other group of non-aborted cows, before the months in which the abortions occurred. This is consistent with authors indicating that the loss of postpartum body conditions seems to affect the course of later pregnancy,<sup>(43)</sup> and that the animals with lower body conditions have higher gestation losses compared to cows with higher body conditions.<sup>(44)</sup> Logistic regression also indicates that body condition variations are protective factors for abortion in months three and four of pregnancy (OR = 0.32,  $P < 0.05$ ).

Total protein levels in both farms were maintained within the RI during the sampling months, and 25 % of aborted cows had low total protein concentrations between months three and seven (Table 4). Studies indicate that total proteins increase

as lactation progresses, and the opposite happens during the dry period, in which there is a slight decrease. According to Piccione et al.,<sup>(33)</sup> there are maternal physiological requirements for supporting fetuses and providing immunoglobulins in milk during the lactation period. Hypoproteinemia can occur in malnutrition cases.<sup>(16)</sup>

Albumin is a negative acute phase protein. Its concentrations decrease in response to injury,<sup>(45)</sup> unlike globulins that increase their levels during the final phase of pregnancy, near calving.<sup>(46)</sup> According to Kaneko et al.,<sup>(47)</sup> these types of proteins are sensitive to nutritional influences when nutrients are low and dehydration processes are elevated. However, these changes are often subtle and difficult to detect and interpret. Plasma globulins increase in inflammatory or infectious processes. In this study, plasma concentrations of albumin and globulin in some abortion cases were associated with inflammatory processes. The herds in general had high values of fibrinogen, which is one of the acute phase proteins that is positive for inflammation, possibly indicating non-specific inflammatory processes.<sup>(16)</sup>

Regarding hepatocellular integrity, reflected in the enzymatic activity of AST, Q3 values in aborted cows exceeded the RI, accounting for damage to the liver and muscles. This triggers a release of bradykinin, histamine, prostaglandins E and F<sub>2α</sub>, causing luteolysis.<sup>(48)</sup> In addition, this hepatocellular damage in aborted cows may be secondary to fat mobilization,<sup>(16, 49)</sup> which was a problem in these herds.

Macromineral metabolites reflect that calcium and phosphorus plasma concentrations in aborted cows were within the RI, although in the herd experienced concentrations under the RI. It has been recorded that cows with serum calcium >2 mM had lower postpartum NEFA serum concentrations than cows with concentrations <2 mM (Reinhardt et al., 2011), indicating that normocalcemic cows have better energy balances than subclinical hypocalcemic cows. Cows are prone to developing alterations in serum concentrations of minerals such as calcium and phosphorus in the beginning of lactation, causing diseases such as hypocalcemia and hypophosphatemia. Nutritional strategies are based on manipulations of endocrine control points by means of the absorption mechanism preparation and resorption of macromineral metabolism, so that cows can more efficiently manage the period of negative mineral balances associated with lactation initiation.<sup>(50)</sup>

Selenium metabolism, estimated by glutathione peroxidase blood activity of aborted and non-aborted cows in both farms, were within the RI, so it could not be inferred that there was a lack of this element throughout gestation in this study. Selenium constitutes one of the essential micronutrients, with an adequate contribution being necessary for health maintenance and the reproduction of ruminants, since many authors show that animals require important levels of selenium during the reproductive stage due to metabolic processes of developing organisms that produce large amounts of free radicals as intermediaries.<sup>(51)</sup>

### *Fat-soluble vitamin plasma concentrations*

Since retinol reflects the contribution of beta-carotene, and given its relevance in the immune response against viruses, its determination was privileged in this trial. Retinol and tocopherol curves during pregnancy of aborted cows did not differ from non-aborted cows ( $P > 0.05$ ). However, a median and negative correlation was found ( $r = -0.42$ ,  $P < 0.05$ ) between retinol plasma concentrations and ELISA results for BVD, which may indicate a protective effect of this vitamin for this disease in the

herds analyzed. According to Weiss, (1998) the concentration of these vitamins in dairy cows depends on feed sources, management systems and feed availability, since the rumen tend to metabolize many of these fat-soluble vitamins.

### *Prevailing abortigenic diseases in the area*

Certain abortions, especially those caused by pathogens, can be “trimester specific”; for example, abortions induced by bovine viral diarrhea tend to occur in the first trimester, abortions due to *Neospora caninum* in the second trimester and those caused by *Leptospira* spp. in the third trimester.<sup>(4)</sup>

The insidious nature of bovine viral diarrhea has generated considerable economic losses to the dairy and beefmeat industries, since infection has been widely disseminated throughout the world. The virus tends to be endemic in most countries with large bovine populations. Accordingly, 60 to 80% of cattle have developed antibodies against this infectious agent, although between 1 and 2 % of the animals are persistently infected.<sup>(52)</sup> It has been noted that aborted cows, as well as cows in the entire study group, were vaccinated against BVD, for which high titres were registered throughout their entire pregnancies. This complicated the inference at the time of infection in the antibody curve, since typically when the animal is exposed to BVDV, it develops a sufficient humoral and cellular immune response.<sup>(53, 54)</sup> However, it was interesting to observe the antibody titres curve over time, since it allowed the identification of critical moments of pregnancy.

This was evident in farm A, where the group of aborted cows recorded a slight rise in antibodies one month after sampling in three and four, where five of eight abortions were recorded, confirming what was recorded for the seroconversion in months three and four. This is consistent with Conrad et al.,<sup>(55)</sup> who stated that animals develop neutralizing antibodies three to four weeks after infection that persist for the rest of the animal's life, although titres decrease with age. On the other hand, no relevant increases were recorded when observing the antibody curve in farm B for the group of aborted cows. However, high antibody titres were maintained for virtually all of the gestation in both farms. This can be explained in part by Saliki and Dubovi,<sup>(56)</sup> who stated that antigen-specific antibodies tend to have greater avidity and higher titres against the specific antigen. Multivariate analysis confirms that serology or seroconversion for BVD in the two premises was not a risk factor, concluding that this disease was not participate in causal mechanisms of abortion at that time.<sup>(57)</sup>

Bovine neosporosis causes abortion between the third month and the end of gestation, and occurs more frequently between the 5th and 6th month of pregnancy.<sup>(58)</sup> This is consistent with the antibody curve determined by ELISA for both farms, in which positive proportions were higher for the aborted cows group ( $P < 0.05$ ), in samples four, seven and eight, which corresponds to field infections, since vaccines were not given to the herds. A total 22.2 % of the aborted cows went through seroconversion for this disease in the samples close to their registry. It is important to determine the titres for this pathogen in pregnant cows, since abortion risks increase when antibody titers are higher,<sup>(59)</sup> which is a good indicator of potential abortion in herds.

As for leptospirosis, there was a greater proportion ( $P < 0.05$ ) of reactive cows in the aborted group (20%), corresponding to Serovars Hardjo and Grippotyphosa

jointly, than in the non-aborted cows. This disease is prevalent in the area, with a 12.2 % of animal prevalence and 52.1 % prevalence in the area,<sup>(20)</sup> evidencing causal patterns of abortion in farm B.

The fact that there were no positive samples for *Brucella abortus* indicates good health farm management. Animals in the region are immunized with the RB-51 vaccine as part of the "National Eradication Plan". This vaccine, applied in high doses, is a safe and stable immunogen that does not cause post-vaccine antibodies, which are detectable in official tests used in Chile for the serological diagnosis of bovine brucellosis.<sup>(60)</sup>

As seen through the probability of abortion analysis, in the time during which gestation and metabolic conditions are interrupted and the serology background for highly prevalent infectious diseases are high in the area, a protective effect ( $P < 0.05$ ) was observed for the gestation of high plasma cholesterol levels and risks ( $P < 0.05$ ) of neosporosis and leptospirosis.

Metabolic imbalances were also observed at the herd level in the dairies analyzed, and different situations of causality were evidenced. In farm A, non-infectious factors predominated (energy imbalances, protein hepatocellular integrity, inflammatory processes), but there were also instances of neosporosis. In farm B, infectious factors predominated, related to leptospirosis and neosporosis. Negative energy balances were recorded in both farms. Thus, cholesterol protection is recommended in the second third of gestation, which, along with BCS evaluation, would be a useful tool for evaluating energy imbalances in dairy herds.

The joint presentation of energy imbalances and leptospirosis or neosporosis is noteworthy. These factors could act as primary or secondary determining factors in the development of abortion, which should be analyzed more in depth in clinical studies.

If the traditional methodology had been followed in the 18 cases of abortions recorded in the study, only routine diagnostic tests for infectious diseases would have been performed, and the ethiological agent would have been suspected in six of them. With the complementary methods applied in the present work, we found a possible association of the remaining cases to metabolic alterations such as energy-protein imbalances at the ruminal level, hepatocellular damage, inflammations, and mastitis.

Abortion, having a multi-factorial causality, is defined as a syndrome;<sup>(4)</sup> therefore, the evaluated variables must be analyzed in certain periods during which the highest incidences of abortions were recorded. Negative energy balances in aborted cows were evidenced by cholesterol concentrations under the RI, BHB increases and low body conditions. It should be considered that the cows in the study were in different trimesters of lactation, which probably resulted in certain variables not registered as significant when included in the logistic regression model.

It must also be indicated that when seasonality was not considered as a source of variation in the study, the effect of heat stress could not be considered,<sup>(61-63)</sup> in countries where increases in temperature are related to reproductive problems in cattle. This can relate to the loss of gestation in the last embryonic stage or in the early fetal stage when the placenta is not yet fully established.

It should also be noted that this work is observational, so the analysis of the variables does not determine causality, but a degree of risk in abortion. However, this background shows the importance of continuing to establish measures for the management and control of infectious diseases that, along with nutritional

imbalances, are associated with the loss of gestation in local dairies. Developing epidemiological surveillance tools to detect these risk factors early can help anticipate reproductive herd losses and improve management of preventive herd medicine. This evidence supports future clinical studies in investigating physio-pathological mechanisms between nutritional imbalances, the development of abortion and predisposition to infectious diseases.

## Conclusions

It was observed that the main metabolic imbalances at the herd level in both dairies analyzed related to energy metabolism, liver integrity and protein metabolism. Aborted cows presented greater alterations in negative energy balances and liver integrity affected by fat mobilization than non-aborted cows, since the aborted cows presented a more marked lack of synchronicity between energy and degradable protein in the rumen, than non-aborted cows.

It is important to help farmers to use epidemiological surveillance tools to prevent reproductive herd losses through management measures, infectious disease control and adequate nutritional management.



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## Data availability

All relevant data are within the manuscript and its supporting information files

## Acknowledgments

We want to thank veterinarian Nicolás Letelier who support in this study, giving us the opportunity to sample the herds

## Funding statement

This research was funded by Fondo Nacional de Desarrollo Científico y Tecnológico (<https://www.conicyt.cl/fondecyt/>), grant number 11110090, awarded to P Gädicke. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Conflicts of interest

The authors report no conflicts of interest, and agree to be held accountable for all aspects of the work. This data has not been published, nor is it under consideration elsewhere.

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