The effect of body condition in the early dry period on the oxidative status of early lactation cows

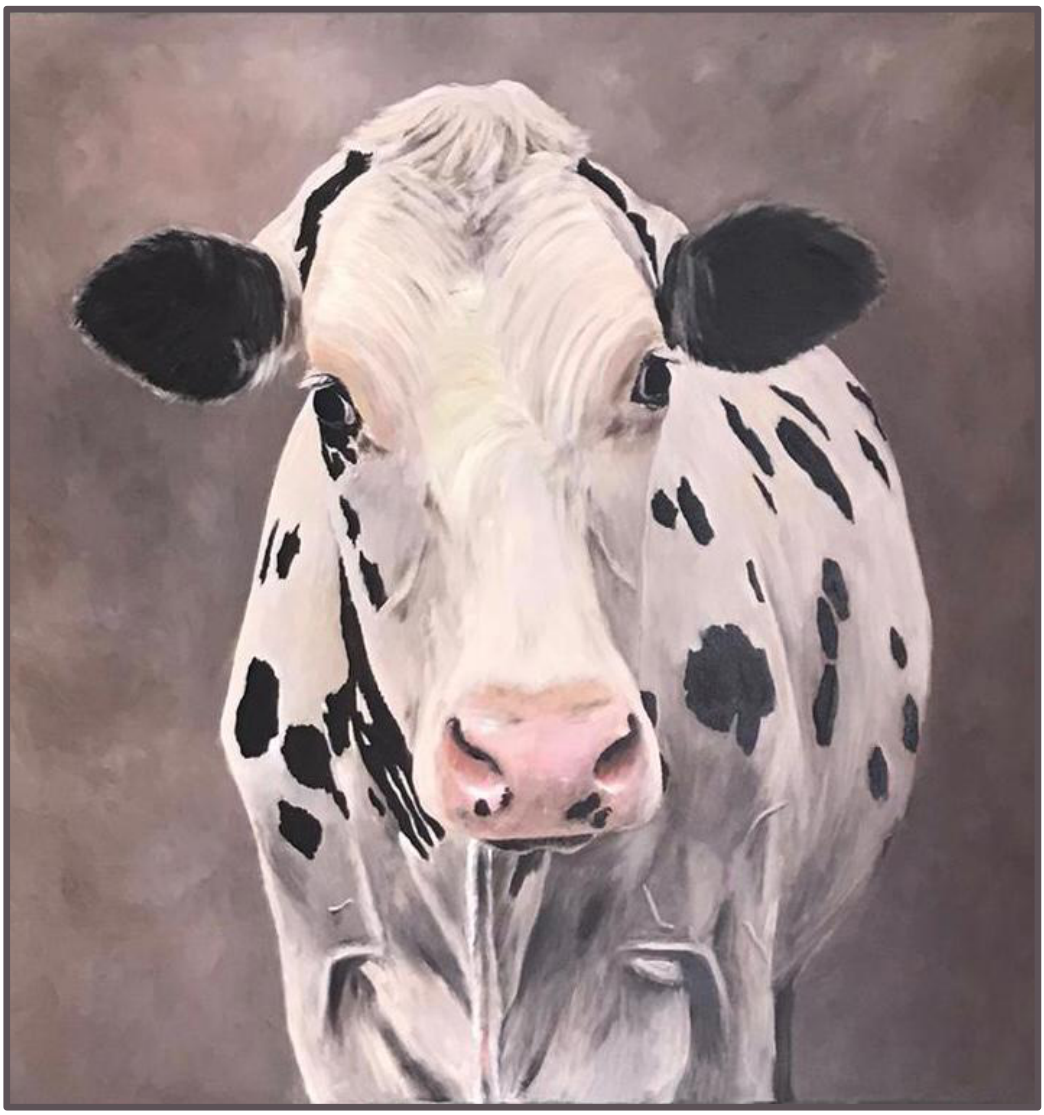

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Table of contents

Abstract

The objective of this study was to investigate if the extent of oxidative damage in early lactation cows is related to high body condition score at the beginning of the dry period. Therefore cows were classified in realistic body condition groups. An existing dataset from a study by Bouwstra et al. (2010) was used, originally investigating the influence of vitamin E supplementation in the dry period on the development of mastitis. In that study, the cows were divided into a low and high vitamin E supplementation group. Body condition was scored and blood samples were taken at five subsequent moments. In this analysis, the cows were classified into three BCS groups: fat, normal and lean condition, using the first moment of body condition scoring at the beginning of the dry period and the sampling moment. These BCS groups were related to the sample results at 18 days post-partum: log serum MDA concentration and the antioxidants and products of oxidative stress serum-alpha tocopherol, albumin, FRAP, ceruloplasmin, uric acid, GSH-px and SOD in erythrocytes. The hypothesis is that cows with a high BCS at the beginning of the dry period will experience a more severe degree of oxidative stress at calving and in the early lactation. The analysis remarkably showed high plasma MDA concentration in the lean condition group at 18 days post-partum. The lean cows that received the high vitamin E supplementation in the original study did not have these high plasma MDA concentrations. Additionally, the analysis suggests that high dietary intake of vitamin E during the dry period might be an effective method to prevent an increase of MDA after calving, even among thin cows.

Introduction

Oxidative stress

When an organism is in a state of aerobic metabolism, reactive oxygen species (ROS) are produced in the mitochondria during the oxidative phosphorylation alias electron transport chain, due to the reduction of oxygen molecules (H. Sies, 1993: H. Sies, 1997). Oxidative phosphorylation is the final step of aerobic metabolism and takes place in the inner membrane of the mitochondria. This membrane contains five proteins, also called complexes. These complexes transport electrons across the mitochondrial membrane. It is this reason that this is also called the electron transport chain. The electrons are used to pump H + from the matrix of the mitochondria to the intermembrane space. The electrons in this chain are coming from the molecules NADH and FADH2, which are products of the glycolysis, fatty acid oxidation and the citric acid cycle. Finally, electrons that have lost their energy are donated to oxygen and formed together with H^+, water. So, in this process oxygen molecules appear to be a crucial part of the electron transfers in this electron transport chain and is the only molecule that can remove electrons from the electron transport chain. The reactivity of oxygen allows the oxygen molecules to be part of the electron transfers. The ultimate product of the oxidative phosphorylation is formed by means of the fifth protein complex , ATP synthetase, which is the well-known adenosine-5-triphosphate (ATP) (Berg et al., 2002; G. J. Burton & Jauniaux, 2011; Hansford, 2002).

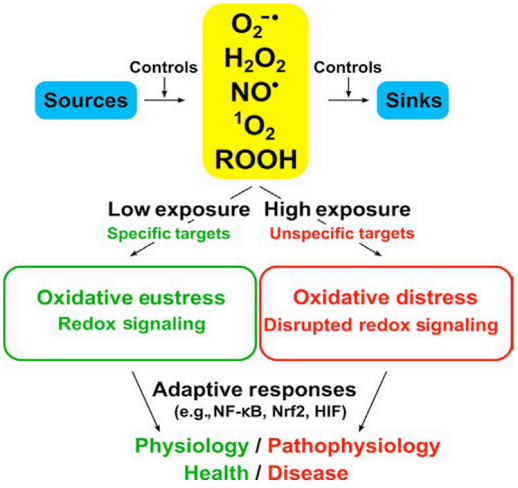

Figure 1: Relationship between oxidative stress and redox signaling (Helmut Sies, 2017).

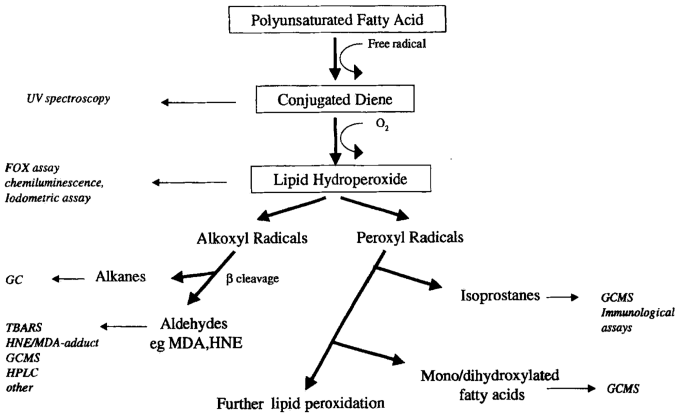

Figure 2: formation lipid peroxidation products (Moore & Roberts, 1998)

ROS are a normal end product of the cellular metabolism. However, a lack of balance between the capacity of the body to scavenge ROS by antioxidative mechanisms and the production of reactive oxygen species can lead to oxidative stress (H. Sies, 1991). The definition of oxidative stress is "*an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage"(H. Sies & Jones, 2007)*. Therefore, a high exposure to oxidants could lead to pathophysiological conditions due to the disruption of redox signaling and biomolecule damage. It is known that ROS can cause peroxidation of lipids, oxidation of cellular macromolecules, proteins and DNA. Unsaturated fatty acids present in e.g., in the lipid bilayer of cellular membranes, are susceptible for oxidation. The susceptibility can lead to a sequence of peroxidation reactions as shown in figure 2. Consequently, immune cells are very sensitive to oxidative stress because the membranes contain high concentrations of fatty acids (Spears & Weiss, 2008). The amount of lipid peroxidation can be quantified by certain biomarkers, which will be explained at the end of this segment.

Oxidation of proteins renders enzymes dysfunctional. Finally, oxidative stress exposure to DNA can lead to mutations, breakage of single or double DNA strands, base misincorporations and can ultimately even cause cell death (Poulsen, 2005).

On the contrary, reactive oxygen species (ROS) in low exposure are needed for proper redox signaling and intracellularly they act as secondary messengers in signaling pathways (G. J. Burton & Jauniaux, 2011). This physiological state of low exposure is called oxidative eustress (see figure 1) (Helmut Sies, 2020). The line between oxidative stress and eustress is still unclear. The term ROS includes free radicals and their non-radical intermediates. Free radicals are molecules containing one or more unpaired electrons. This unpairedness of electrons in the electron shell provides the high reactive power of radicals.

The superoxide anion (SO), produced by the mitochondria, is the most abundant and initial oxygen radical (Cadenas & Davies, 2000). Furthermore, SO is formed by physiological as well as pathological processes. One of the ways SO anions arise is when the transfer of electrons in the electron transport chain (also known as the respiratory chain) do not go well. As a result, electrons leak to oxygen molecules. How many SO anions are formed depends on the metabolic status of a dairy cow. The more oxygen is used in aerobic metabolism, the more electrons are transported in the electron transport chain, resulting in more SO anions being produced. However, in case of a shortage of oxygen molecules, electrons can accumulate in the chain which can also lead to an increased number of SO anions. Under physiological conditions, about two percent of oxygen will be converted to SO anions in the mitochondria. The SO anion molecule is charged and therefore cannot cross the cell membrane and will remain in the mitochondria (G. J. Burton & Jauniaux, 2011). Additionally, SO anions can also be formed in the shorter electron transport chain in the endoplasmic reticulum (ER). In the ER, protein folding takes place under the influence of oxygen. This is an oxidative process which causes the release of electrons, that in turn, cause SO anions to be formed (G. J. Burton & Jauniaux, 2011; Tu & Weissman, 2004). Furthermore, the enzymes NADPH-oxidase, 5 cytochrome P450 and other oxide-reductases contribute to the production of SO anions (G. J. Burton & Jauniaux, 2011). Superoxide anion is converted by the enzyme superoxide dismutase in hydrogen peroxide and oxygen (Y. Wang et al., 2018).

In the periparturient period, when dairy cows go from gestation to lactation, cows undergo important metabolic changes. There will be an increase of metabolic activity and this can result in a higher amount of oxygen consumption, which is associated with an increased release of ROS (Barry Halliwell & John M. C. Gutteridge, 2015). Consequently, cows might suffer from increased oxidative stress in this transition period. Indeed, Lohrke et al. (2004) found a relationship between milk yield per day and hydroperoxide (indicator for oxidative stress). Several studies have reported that early lactation cows are at risk for oxidative stress (Bernabucci et al., 2005; Gaál et al., 2006; Konvičná et al., 2015; Liu et al., 2013), but some authors reported no serious oxidative stress in this period (Dobbelaar et al., 2010; Wullepit et al., 2009). The study of Gong & Xiao (2016) has shown that early lactation cows (day 1 till day 30) have more severe oxidative stress, serum GSH-Px and malondialdehyde (MDA), measured with colorimetric assay kits, were both increased. This certainly shows that in the transition period of dairy cows, an increase of oxidative stress can occur. Sordillo & Aitken, (2009) suggest that the increase of oxidative stress in transition cows is the cause for disfunction of inflammatory responses.

Direct measurement of ROS is difficult because of the short half-lives of ROS. Therefore, ROS can’t be measured in cows. An alternative way to quantify the nature and degree of oxidative

stress is to measure parameters which are created by the reaction of ROS with biomolecules (Ghezzi et al., 2020). One of these biomarkers is malondialdehyde (MDA) which is originated by peroxidation of polyunsaturaded fatty acids (PUFAs) (Placer et al., 1966; Tejero et al., 2007) and can be used to quantify oxidative stress (Jamali Emam Gheise et al., 2017; Nielsen et al., 1997). MDA can be measured by thiobarbituric acid reacting substances test (TBARS), but it can also be measured by high-performance liquid chromatography (Lykkesfeldt & Svendsen, 2007).

Antioxidants

To counter the damage caused by ROS, the body has several enzymatic and non-enzymatic protection mechanisms. The concentration of antioxidants such as vitamin A (beta carotene), selenium, glutathione and alpha-tocopherol in blood and tissues are also a good benchmark for the degree of oxidative stress (Ighodaro & Akinloye, 2018). Besides, another way to obtain an indication of the degree of oxidative stress is to measure the quantity of antioxidative enzymes such as glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) (Bernabucci et al., 2005; Gaál et al., 2006; Ighodaro & Akinloye, 2018; Lubos et al., 2011).

Enzymic antioxidants

The initial line of defense against peroxidative damage is comprised of the three enzymes catalase, glutathione peroxidase and superoxide dismutase (McDonald et al., 2011). Superoxide dismutase takes place in the initial line of antioxidant defense by elimination of, as its name suggests, superoxide radicals. Catalase is an antioxidant mainly located in the peroxisomes, where beta oxidation takes place. Its antioxidant function also relies on the reduction of H_2O_2 to water and oxygen (Glorieux & Calderon, 2017). Particularly, the enzymic antioxidant glutathione peroxidase is the major intracellular antioxidant defense (Sen et al., 1994). It catalyzes the reduction of H_2O_2 to water and oxygen as well as the reduction of peroxide radicals to alcohol and water.

Additionally, it is known that the ferroxidase ceruloplasmin is an antioxidative biomarker (Inoue et al., 2013; Mzhel'skaya, 2000), its antioxidant function being the oxidation of toxic ferrous iron into non-toxic ferric iron.

Non-enzymatic antioxidants

Below non-enzymic antioxidants relevant for dairy cows will be discussed.

Albumin is an important plasma protein that can bind and scavenge free radicals directly (M. Roche et al., 2008). Its abundancy and outstanding binding capacity qualify it as an effective anti-oxidant.

Thiols are an example of the importance of the cooperation of all antioxidants, being an important component of the function of peroxidases, as they use thiol-based reactions to detoxify H_2O_2 (Ulrich & Jakob, 2019).

Uric acid circulates the bloodstream scavenging reactive radicals, and can prevent lipid peroxidation, inasmuch as ascorbic acid is also present (Sautin & Johnson, 2008).

Dietary antioxidants
There are several vitamins and trace minerals that are known to contribute to the antioxidant defense system.

Vitamin E
One of those vitamins is vitamin E and it has been shown that it influences the health of cows by lowering oxidative stress and positively contributing to the immune system (G. Burton & Traber, 1990; Spears & Weiss, 2008). Vitamin E consist of eight different forms (Obranović et al., 2015): α-, β-, γ- and δ-Tocopherol, α-, β-, γ- and δ-Tocotrienol. All of these forms are derived from 6-chromanol and all are lipid soluble (Herrera & Barbas, 2001). α-tocopherol is the most biologically active (Oram et al., 2001). The antioxidant capacity of tocopherols is facilitated by their lipid solubility in combination with the ability to break the chain reaction of lipid oxidation by forming stable chromanoxyl radicals (Obranović et al., 2015). This is done by scavenging peroxyl radicals and donating their phenolic hydrogens (Kamal-Eldin, 2019). Its localization in cell membranes protects the oxidation of unsaturated fatty acids and ensures membrane integrity is maintained(G. W. Burton et al., 1983; Traber & Atkinson, 2007; X. Wang & Quinn, 1999). The recommended plasma vitamin E concentrations in cows around calving is > 3 µg/ml (Politis, 2012; Weiss et al., 1997).

Selenium
Another important ingredient that cows get from their diet is the trace element selenium. Selenium is needed for peroxidase enzymes such as the cytosolic glutathione peroxidase enzyme (GSH-px). Besides, it also of great importance for the thyroxin- and arachidonic acid metabolism (Ivancic & Weiss, 2001).

Beta carotene
Beta carotene is the precursor for retinol (Vitamin A) and is an important antioxidant due its neutralizing capacity of radicals (Helmut Sies & Stahl, 1996).

Pro-oxidants
Opposite to the above described antioxidants, there are also substances which may act as pro-oxidants. These are endo- or xenobiotic substances that induce oxidative stress by either generating ROS or inhibiting the above described antioxidants (Rahal et al., 2014)
Two relevant examples of this are a dietary excess of iron resulting in a so-called iron overload and PUFAs. Free iron is redox reactive and therefore catalyzes the formation of ROS (Cheng & Lian, 2013). PUFAs are a bioactive nutrient that is especially vulnerable to free radical oxidation (Bouwstra et al., 2009; Di Nunzio et al., 2011).

Relationship Body Condition Score and oxidative stress
Researchers found that humans with more visceral fat have more oxidative damage (Palmieri et al., 2006; Savini et al., 2013; Skalicky et al., 2008). Fernández-Sánchez et al. (2011) explain the different mechanisms by which obesity leads to oxidative stress. Not only the production of ROS through oxidation of fatty acids in mitochondria and peroxisomes, but also by overconsumption of oxygen, releasing free radicals in the mitochondria. Furthermore, the increase of adipose tissue in obese humans causes a significantly decreased function of several antioxidant enzymes.

With regard to cows, the amount of body fat is usually estimated by the Body Condition Score (BCS). The study of Bernabucci et al. (2005) has demonstrated that cows with a high BCS at the moment of calving have more BCS losses and have a more conspicuous change of oxidative

status. They measured parameters of oxidative stress in plasma during early lactation. They found that cows in the high BCS group had significantly higher plasma ROM, TBARS, and plasma SH and lower erythrocyte SH and SOD, compared to the cows in the medium and low BCS groups (Bernabucci et al., 2005). Nonetheless, Kilk et al. (2014) have studied the validity of the d-ROM test. They conclude that the d-ROMs-test is not the most accurate way to quantify the degree of oxidative stress as various serum components can interfere with this test. Additionally, Spears & Weiss, (2008) describe how lipid peroxidation is initiated by ROS and thereby harm tissue by cell damage.

However, in this study the researchers used questionable interpretation of BCS standards. Cows with a BCS lower than 2.5 were assigned a low body condition score, cows with a BCS between 2.6 to 3.0 were assigned a medium body condition score and cows with the body condition score of 3.0 or higher were assigned a high body condition score. This scoring system was used according to the Agricultural Development and Advisory Service (U.K.) (1986). It is questionable if the cows with a BCS of 3.0 or higher are rightly labeled as cows with high body condition. Moreover, Dobbelaar et al. (2010) also suggest that cows with a BCS of 3 or higher 4 weeks before calving, are prone to more peroxidative damage during the early lactation. This study used the scoring system developed by Ferguson et al. (1994). Furthermore, this study labeled cows with a BCS of 3 or higher as high body condition score, which also makes these results questionable.

Changes in BCS

As previously mentioned, in the periparturient period, dairy cows experience metabolic changes. This is because the suddenly rising energy requirements for the increasing milk production cannot be met by the consumption of feed. To compensate this shortage of energy during the so-called negative energy balance, fatty acids are mobilized from the adipose tissue. These fatty acids are metabolized in the liver through the earlier mentioned process of lipid peroxidation, an aerobic process where ROS are generated. In other words, the increased mobilization of fatty acids is associated with the increase of aerobic metabolism and thus the formation of radicals (Bernabucci et al., 2005). Measuring the mobilization of fatty acids could therefore be an indicator of a dairy cow's oxidative status. Parameters for fatty acid mobilization are non-esterified fatty acids (NEFAs) and BHBA (van der Drift et al., 2012).NEFAs are a direct indicator of lipolysis as they are the free fatty acids mobilized from the adipose tissue to be metabolized in the liver. BHBA is a product of ketosis and a biomarker for subclinical ketosis, a metabolic condition that occurs when the liver is overwhelmed with NEFAs (McArt et al., 2012).

The aim of the study

The aim of this study is to investigate if the extent of oxidative damage in the early lactation is related to a high body condition score at the beginning of the dry period. In this analysis we use an existing dataset from a field study conducted in the Netherlands (see Bouwstra et al., 2010). Bouwstra et al. (2010) primarily investigated the effect of vitamin E supplementation on the probability of cows developing mastitis. In that study the body condition was scored at five subsequent moments. In this analysis we will use the first moment of body condition scoring, which is at the beginning of the dry period, and relate this with log serum MDA concentration.

Our hypothesis is that cows with a higher BCS in the beginning of the dry period will experience a more severe degree of oxidative stress at calving and in early lactation.

Material and methods

This analysis used an existing dataset from a field study conducted in the Netherlands. In this study, the researchers studied the relationship between vitamin E supplementation on the occurrence of mastitis (Bouwstra, Nielen, Stegeman, et al., 2010). This study was described in Bouwstra et al. (2010) and Dobbelaar et al. (2010). The following details are derived from the aforementioned papers.

Farms and animals
Five commercial dairy farms were selected in the Netherlands. The farms were selected on the following criteria. Firstly, all the farms had a minimum of 100 dairy cows. Secondly, the farms had a mean 305-days-production of between 9000 kg and 9500 kg milk yield. Furthermore, over 12 months, the farms had a minimum clinical mastitis incidence of at least 15% per month or 30% per year. The housing of the animals was comparable on all farms. The feeding and thus the composition of rations differed per farm. However, all rations existed of grass silage and maize silage. Prior to this study, two farms (A and E) fed their lactating cows fresh grass.

Experimental groups
The experimental groups are composed as follows: cows and heifers were randomly assigned to one of two treatment groups after dry-off. Both groups received vitamin E (dl-alpha-tocopheryl-acetate) as part of a mineral mixture in their feed. Treatment-group 'A'/'LE' received 135 IU/day dl-alpha-tocopheryl-acetate added to their feed and treatment-group 'B'/'HE' received 3000 IU/day dl-alpha-tocopheryl-acetate. The treatment groups were housed separately and fed the same rations, except for the different concentration of dl-alpha-tocopherylacetate. The researchers and the farmers were blinded for the treatment groups. Finally, 43 cows that had clinical mastitis were excluded from this analysis because it was supposed that their capacity to regenerate alpha tocopherol was disturbed.

Measurements and sampling methods
At five different moments blood samples were taken from the cows. The samples were taken in a lithium heparin tube, a sodium fluoride/potassium oxalate tube and serum separator tubes. The samples were all collected from the vena jugularis with the use of a vacutainer-system.

Table 1: Mean days ante- or post-partum per blood sample number

Sample number	Days*	N	Standard deviation
1	- 54,2	283	12,4
2	- 25,7	287	6,8
3	-12,2	275	6,4
4	0,83	250	0,78
5	18,9	283	8,5

* '-x' = days ante partum

The blood samples were, except for sample number 3, were taken in the morning between 8h and 12h. After sampling, the samples were transported to the laboratory. During the transportation, the samples were held under the condition of a minimum of 6 degrees Celsius and maximum of 16 degrees Celsius.

Both α-tocopherol and MDA were measured through an isoratic HLPC system by using a kit from Chromsystems (Munich, Germany). GSH, GSH-Px, GSSG and Hemoglobin were analyzed with an autoanalyzer (Hitachi 912). The method described by Benzie & Strain. (1996) was used for the measuring of the FRAP antioxidant assay. Uric acid, BHBA and NEFAs were measured using a clinical auto-analyzer (LX-20 Beckman-Coulter, Woerden, The Netherlands) Finally, all the analyses were carried out at the National Institute for Public Health and Environment.

Body Condition Score

The BCS was noted at the same moment the blood samples were collected. The study used the scoring system developed by Ferguson et al. (1994). For this analysis, cows were classified in the following BCS groups:

- Lean condition: BCS < 2,5
- Normal condition: BCS > 2,5 - < 3,5
- Fat condition: BCS > 3,5

Statistical analysis

For the statistical analyses IBM SPSS Statistics version 25 is used (IBM Nederland BV, The Netherlands, Amsterdam). An effect was considered significant when the p-value was < 0,05. First of all, normality was tested by using the Shapiro-Wilk statistic. Log10 transformations were used if necessary, to achieve normal distribution. This was done on glutathione, NEFA, GSSG, malonaldehyde and α-tocopherol. Additionally, body condition scores were divided into 3 groups. This was convenient to plot the BCS against oxidative stress parameters and antioxidants. The cows were divided over three groups (BCSK: body condition score klasse), BCSK 1: BCS < 2,5, group BCSK 2: BCS > 2,5 - < 3,5 and group BCSK 3: BCS > 3,5.
Only the early lactation sample has been analyzed and, as only clinically healthy cows were used in this analysis to prevent interference of inflammation, cows with mastitis were eliminated from the dataset.

The data was analyzed using a general linear model univariate analysis. The dependent variable in this analysis was log serum MDA and the fixed factor was the BCSK. The covariates were SOD, albumin, ceruloplasmin, GSH-px-Hb, alpha-tocopherol, FRAP and uric acid. Covariates were divided in proteins or non-proteins. A backward procedure was used to reduce the number of independent variables in the model.

Results

After elimination of cows that suffered from mastitis, 236 cows remained for analysis.
For unknown reasons, 6 cows did not have a BCS at the first sample moment. Additionally, one cow from the lean group has no result for MDA and alpha-tocopherol concentration. Also, one cow from the normal group has no result for alpha-tocopherol.

Descriptive statistics

The frequency distribution of the BCSK cows is shown in table 2.

BCSK	N = Cows
1 (BCS < 2,5)	63
2 (BCS = 2,5 - 3,5)	88
3 (BCS > 3,5)	79
Total	230

Table 2: Distribution body condition score at the beginning the dry period (scored on average 54 days before calving)

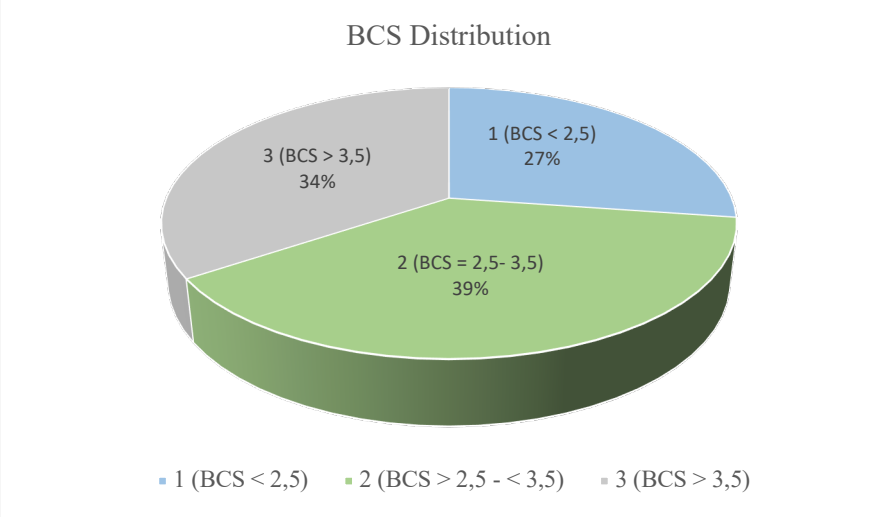

Figure 3: Distribution body condition score at the beginning the dry period (scored on average 54 days before calving)

FFA (figure 4) and BHB (figure 5) are plotted in a chart to demonstrate the development of fatty acid mobilization reflecting the metabolic status. Both charts show that all three BCSK groups experience roughly the same metabolic changes. There are differences between the BCSK groups regarding the FFA and BHBA concentration, but only FFA is significantly lower for the cows in BCSK 2 (P = <0,05).

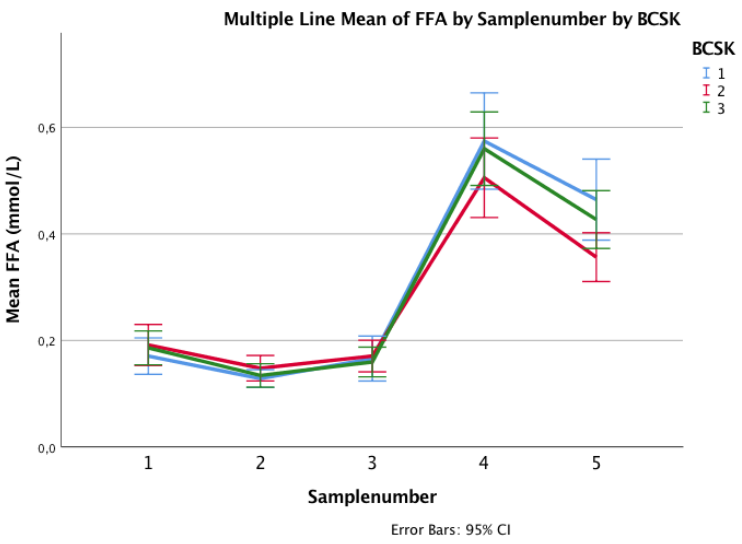

Figure 4: Multiple line means of free fatty acid (mmol/L) by sample number by BCSK

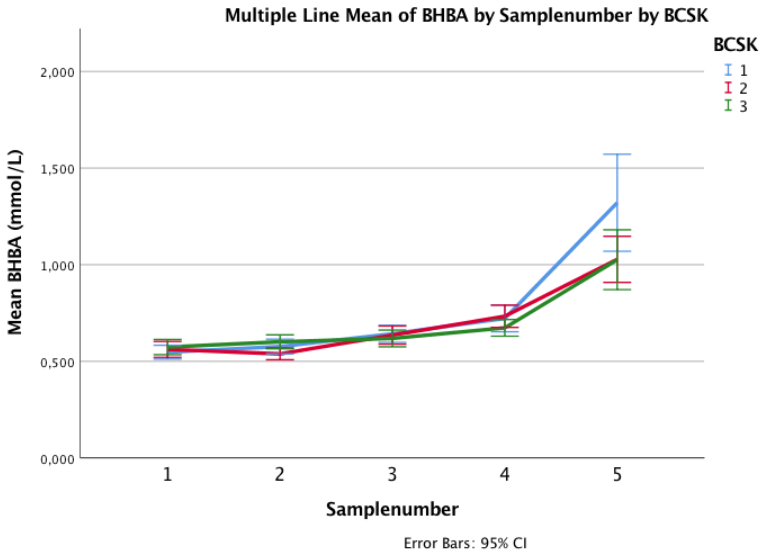

Figure 5: Multiple line means of beta hydroxybutyrate (mmol/L) by sample number by BCSK

Means of the variables SOD, GSH, MDA, albumin, ceruloplasmin, GSH-px-Hb, alpha-tocopherol, FRAP and uric acid were plotted in a chart against the five sample moments. The lines of SOD, albumin, ceruloplasmin, GSH-px-Hb, alpha-tocopherol, FRAP and uric acid followed a similar pattern for the three different BCS-groups (see appendix 1). As shown in figure 6, the cows in the high body condition group have on average the lowest concentration serum MDA over the entire period. Both the normal condition cows and the lean condition cows have a higher average serum MDA concentration.

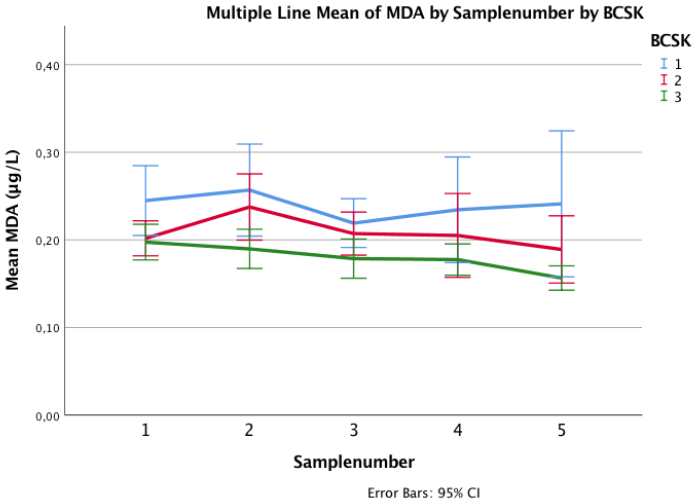

Figure 6: Multiple line means of MDA by sample number by BCSK

The difference with regard to MDA between the treatment groups from the study of Bouwstra et al. (2010) was also examined. For treatment group A (N= 124), the cows on a low vitamin E supplementation, the mean MDA of the three BCSK groups at the different sample moments is shown in figure 7 Here, the lean condition group shows a considerable increase in mean MDA around calving. Figure 8 shows the mean MDA of the three BCSK groups at the different sample moments for the cows in treatment group B (N=112), with a high vitamin E supplementation. The line of the lean condition group considerably drops from sample number 2, similar to the other two condition groups.

The interaction between BCSK and alpha-tocopherol was tested, but there were no significant interactions between BCSK and alpha-tocopherol (P> 0,05) .

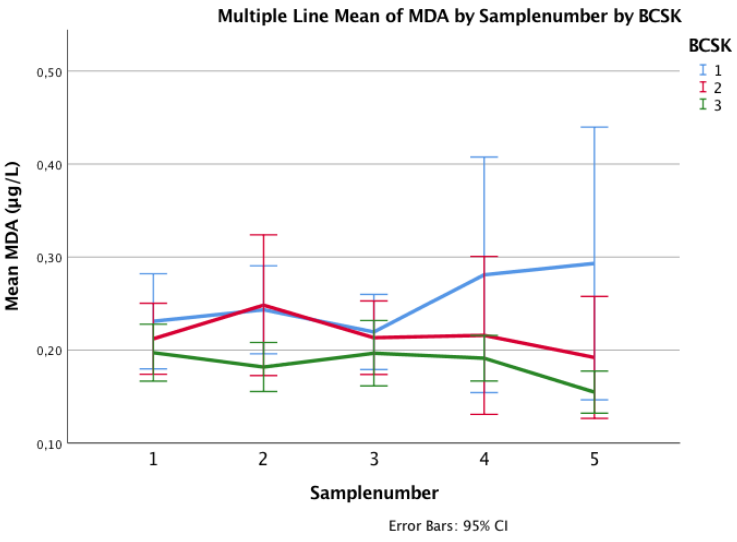

Figure 7: A; Multiple line means of MDA by sample number by BCSK on the low vit E level

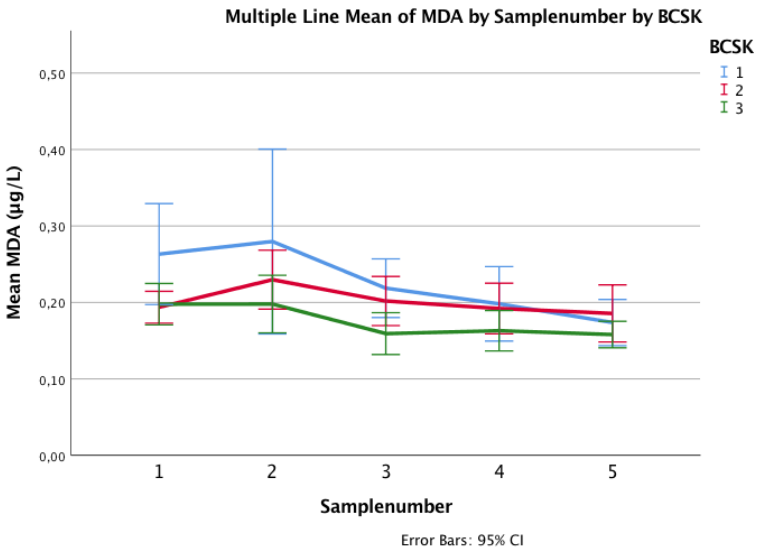

Figure 8: B; Multiple line mean of MDA by sample number by BCSK on the high vit E level

For sample number 4 and 5 it has been questioned whether there was an association between BCSK and oxidative stress. Means of the variables SOD, GSH, MDA, albumin, ceruloplasmin, GSH-Px/gHb, alpha-tocopherol, FRAP and uric acid from sample number 5 are shown in Table 3 (for sample number 4, see appendix 3).

BCSK		BHB (mmol/L)	MDA (µg/L)	Albumin (g/L)	FFA (mmol/L)	CP (g/L)	FRAP (µmol/L)	GSH-Px/gHb (gr/Hb)	SOD (U/g Hb)	URIC acid (mmol/L)	alpha-tocopherol (µmol/L)
1	**Mean**	1,321	0,241	34,452	0,464	729,815	257,276	4,805	42,679	65,865	12,299
	N	63	62	63	63	63	63	63	63	63	62
	SD	0,997	0,327	2,900	0,302	138,043	54,095	1,359	13,311	12,712	4,701
2	**Mean**	1,028	0,189	35,585	0,356	746,485	271,756	4,890	42,275	69,807	13,450
	N	88	88	88	88	88	88	88	88	88	87
	SD	0,562	0,181	3,002	0,216	135,314	52,557	1,221	11,810	10,668	4,460
3	**Mean**	1,026	0,156	35,915	0,427	727,033	284,610	4,871	46,312	71,486	12,670
	N	79	79	79	79	79	79	79	79	79	79
	SD	0,691	0,062	3,187	0,242	124,748	63,636	1,546	16,302	12,176	4,161
Total	**Mean**	1,107	0,192	35,388	0,410	735,238	272,205	4,860	43,772	69,304	12,867
	N	230	229	230	230	230	230	230	230	230	228
	SD	0,754	0,209	3,084	0,254	132,272	57,753	1,372	13,960	11,937	4,434

Table 3: concentrations sample number 5 variables per BCSK group

Univariate analysis

The univariate analysis was done for sample number 4 (calving) and 5 (early lactation). For sample number 4 no significant correlations were found.

Sample number 5

The dependent variable was log serum MDA and BCSK was a fixed factor. The covariates were SOD, albumin, ceruloplasmin, GSH-px-Hb, alpha-tocopherol, FRAP and uric acid. Covariates were initially grouped into proteins or non-proteins. SOD, albumin, alpha-tocopherol, FRAP and uric acid were all not significant associated with log serum MDA. The remaining variables from both protein and the non-protein group were combined in the univariate analysis. BCSK, GSH-px-Hb, log GSH and ceruloplasmin were significantly correlated with log serum MDA.

Source	Type III Sum of squares	df	Mean square	F	Significance
Corrected model	1,468	5	0,294	7,917	0,000
Intercept	0,187	1	0,187	5,032	0,026
Ceruloplasmin	0,383	1	0,383	10,316	0,002
GSHlog	0,148	1	0,148	3,998	0,047
GSH-Px-Hb	0,337	1	0,337	9,085	0,003
BCSK	0,336	2	0,168	4,531	0,012
R Squared = 0,151 and adjusted R squared = 0,132					

Table 4: Correlations between log serum MDA and BCSK, GSH-px-Hb, log GSH and ceruloplasmin

With 'farms' as fixed factor in the model the R squared was 0,302.

Discussion

The aim of this study was to evaluate the effect of the BCS at the beginning of the dry period on the extent of oxidative damage at calving and in the early lactation. This was measured by only one biomarker, MDA. The hypothesis was that cows in the fat condition group would experience a more severe degree of oxidative lipid peroxidation at calving and/or in the early lactation.

Metabolic status

The results show that the cows in the lean group have higher plasma NEFAs and BHBA in the sample 18 days post-partum, although only the plasma NEFAs are significantly different. Regarding BHBA, the fat condition group has the lowest concentration and with regard to NEFAs, the normal condition group has the lowest concentration. These results contradict the expectation that high body condition cows would have the highest amount of fatty acid mobilization (J. R. Roche et al., 2013) and would therefore be most compromised by the negative energy balance. However, the level of plasma NEFA remains below 1,0 mmol/L, suggesting that the degree of fat mobilization was not excessive in all three BCSK classes.

The early lactation sample analyzed in this study was taken on average 18 days post-partum. Although this is indeed in the early lactation, it might be too early in the early lactation to draw conclusions about the metabolic status. There is evidence that suggests that later in the early lactation, cows still suffer from negative energy balance. To illustrate this van der Drift et al. (2012) described the course of plasma BHBA and NEFA concentration from 4 weeks ante partum until 8 weeks post-partum. They showed that BHBA was highest at 6 weeks post-partum. Akbar et al. (2015) confirmed this when comparing NEFAs and BHBA between cows of high, medium and low BCS at calving. Plasma concentration of NEFAs was highest in the high BSC group, followed by medium and low condition, respectively. Plasma NEFA and BHBA concentrations peak in week 3 post-partum and are still elevated at the end of the study, 6 weeks post-partum. Rastani et al. (2005) even describe negative energy balance in fat condition cows until 10 weeks post partum. These results show that although the negative energy balance peaks around three weeks post-partum, it may occur over a prolonged period of time for up to 10 weeks post-partum. Sampling at 18 days post-partum is an early moment in the lactation, just before the average peak of negative energy balance. Therefore it is possible that the cows in this study would experience a comparable course of negative energy balance. Figure 5 in this study suggests to confirm this concept as it implies a still increasing plasma BHBA concentration. The plasma concentration of NEFAs or, in figure 4 called FFA, is already decreasing but is still elevated at 18d post-partum. Thus, these results neither fully support nor reject the hypothesis of this study.

Oxidative status

The only sample in the beginning was taken on average 18 days after calving. A further increase of MDA later in early lactation was reported (Knoblock et al., 2019).

The outcome of the descriptive statistics was remarkable as, contrary to the hypothesis, it showed higher serum MDA concentrations in the lean body condition group compared to the normal and fat condition groups. After comparison between the two treatment groups from the original study this dataset was created for, it was clearly demonstrated that the lean cows in treatment group A (low vitamin E supplementation) had an increased mean plasma MDA concentration in the post-partum sample, whereas in the other BCSK, plasma MDA concentration decreased. In high vitamin E treatment group B, the lean cows had a decrease of MDA in the early lactation sample, along with the two other BCSK groups. This suggest that

in this study, lean cows without supplementation of vitamin E during the dry period undergo more oxidative stress than normal or fat condition dairy cows. The fact that the fat and normal condition cows do not have an elevated plasma MDA concentration when given a low vitamin E supplementation, implies that these cows might have vitamin E reserves stored in the body fat. This idea can be supported by the fact that vitamin E is lipid-soluble (Herrera & Barbas, 2001) and literature describing that vitamin E can indeed be stored in adipose tissue (Rizvi et al., 2014). Ross et al. (2014) even described that in humans, 90% of the body's vitamin E content is stored in the adipose tissue. These reserves may then be mobilized along with the fatty acids in periods of shortage like the periparturient negative energy balance. However, no evidence on this and the mechanism by which this should happen is available. Moreover, it is unclear how this will develop over the course of the negative energy balance. Will plasma MDA remain low for fat and normal condition cows or will it eventually rise when this hypothetical vitamin E reserve runs out? This would need to be examined in further research.

Does the unexpected outcome of the analysis mean that the hypothesis must be rejected? No, since there are several explanations why the hypothesis might still apply. Helmut Sies (2020) describes how several biomarkers are potentially linked to oxidative stress. MDA, as tested significantly in this study, is one of those biomarkers. dROM is also still considered as a marker of oxidative stress, and therefore was measured in this study. However, no significant correlations were found. Moreover, several authors state that dROM is not a proper marker for oxidative stress (Dobbelaar submitted; Kilk et al., 2014) as other serum components, like ceruloplasmin, interfere with the test. Therefore, it was decided that MDA would be the only accurate marker of lipid peroxidation.

Moreover, multiple antioxidants were statistically analyzed. Table 3 shows that ceruloplasmin, GSHlog, GSH-px and BCSK are all significant predictors of log serum MDA. Thus, these variables contribute significantly to the degree of the MDA concentration. The R squared value is 0,151 which means that only 15% of the variation in our MDA can be explained by this statistical model. Even though MDA is significantly predicted by the values of ceruloplasmin, GSHlog, GSH-px and BCSK, the low R-value renders the predictive value small. This suggests the predictive value is significant, but weak. A low R-value can be a consequence of wide variety in the data, as clearly illustrated by the wide 95% confidence intervals in figure 7. The R-value can be increased by increasing the sample size. However, when farms are included in the univariate analysis, with 'farms' as fixed factor, the R Squared doubled. In this study, this would give a questionable outcome, as the characteristics of the farms are unknown.

The correlation of GSHlog and GSH-px to MDA was significant, although weak. GSH-Px is an antioxidative enzyme that depends on the Se intake for a longer period. This finding confirms its role as antioxidant and put emphasis on the importance of an adequate Se status prior to calving.

Conclusion

Contrary to the hypothesis, cows in the fat body condition group do not experience a more severe degree of oxidative stress in the early lactation irrespective the level of vit supplement in the preceding dry period. At least, this does not apply to dairy cows as early as 18 days post-partum. Literature suggests that later on in the early lactation, between 3 and 6 weeks post-partum, cows experience peak negative energy balance in terms of plasma NEFA and BHBA concentration. Plasma NEFAs, BHBA and MDA can still be elevated as late as 8 weeks post-partum. This evidence implies that, had this study collected data for a longer period of time, the results might have been different.

The possibility of vitamin E deposits in the adipose tissue as a reserve among fat and normal condition cows needs further and longer research in order to be fully supported. An adequate dietary intake of selenium and vitamin E during the dry period might be an effective method to prevent an increase of MDA after calving, even more among thin cows.

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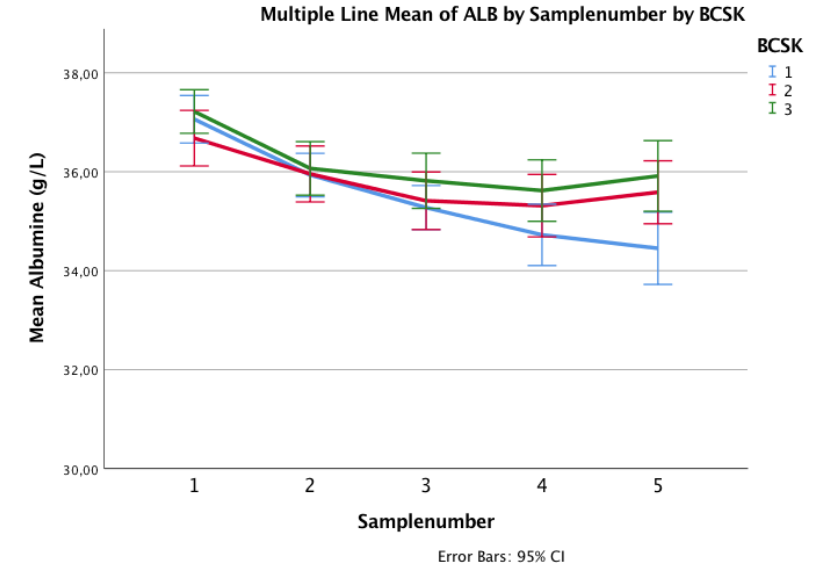
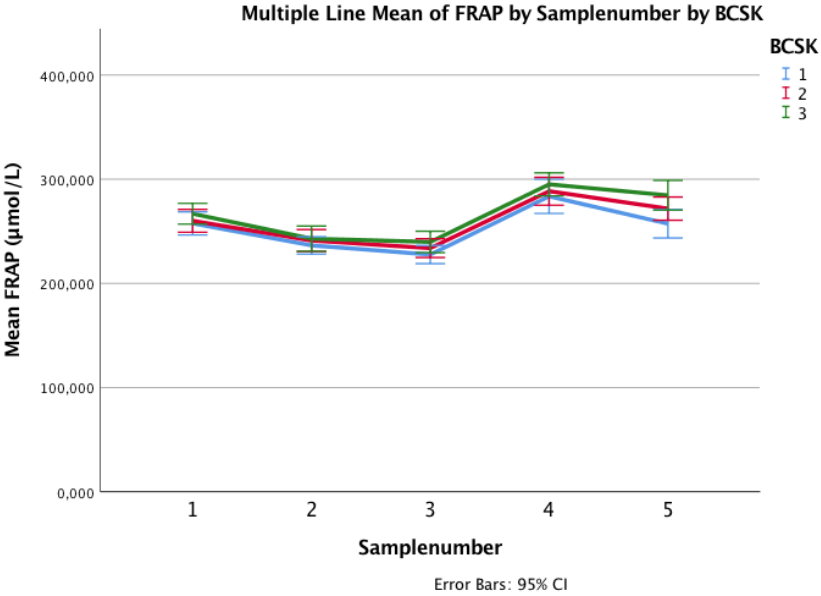
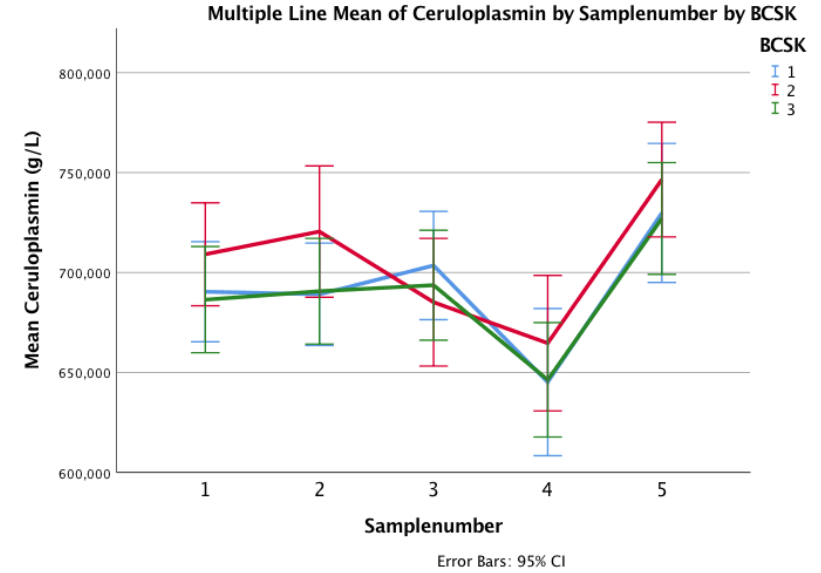
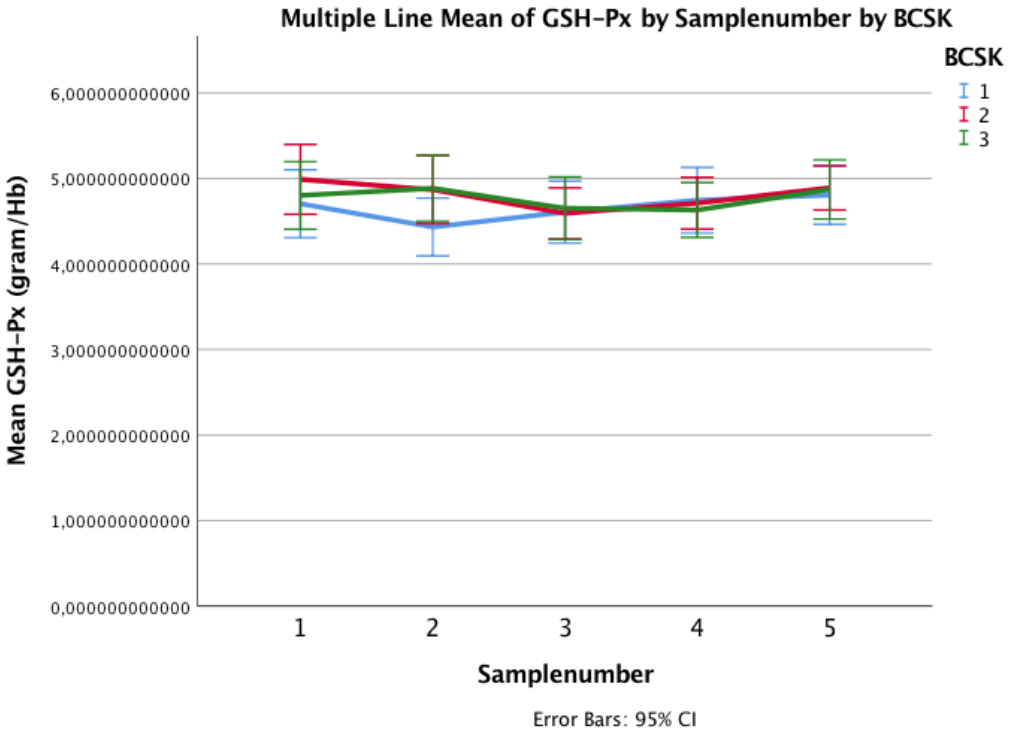
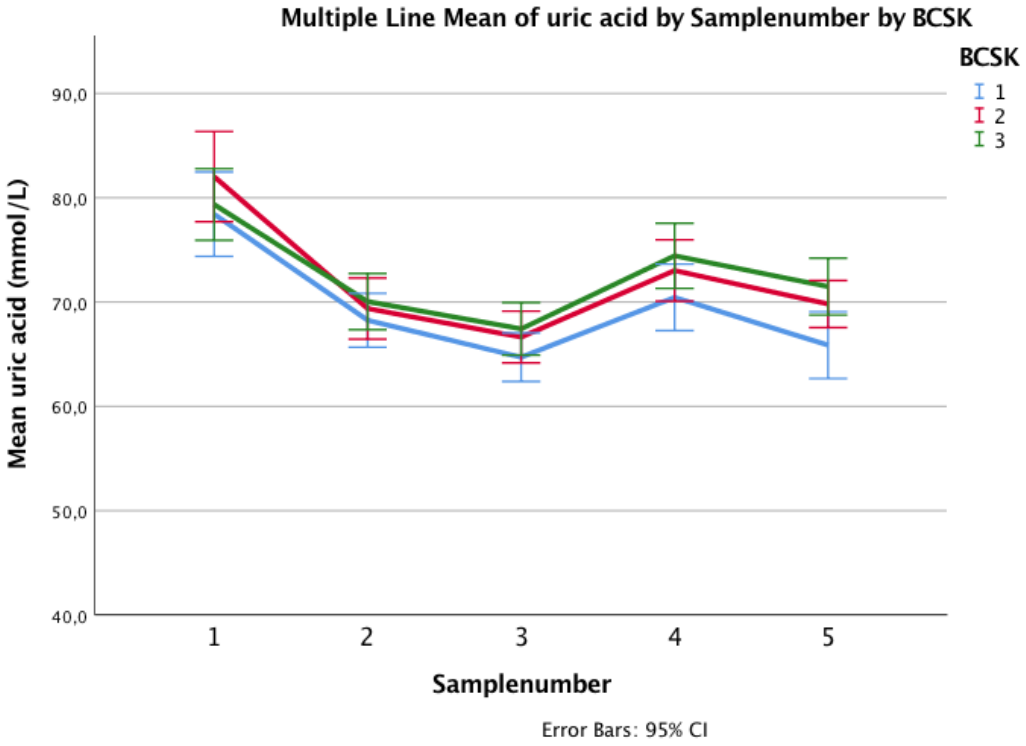
Appendix 1: Line graphs

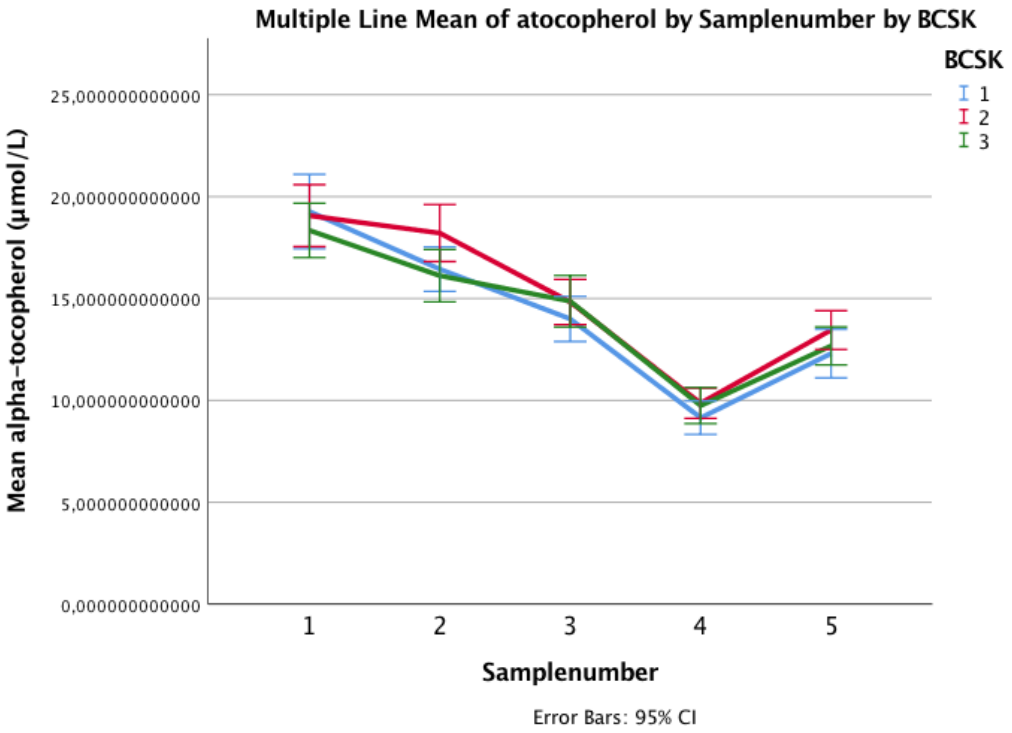


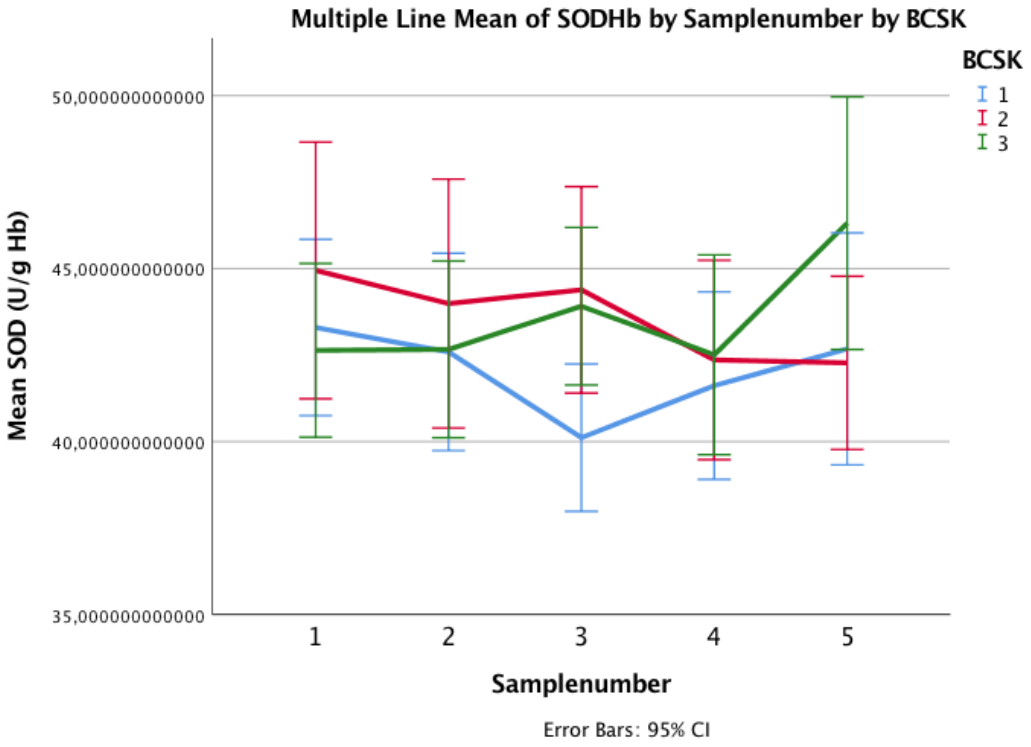





Multiple Line Mean of GSH-Px by Samplenumber by BCSK
BCSK
1
2
3
Mean GSH-Px (gram/Hb)
6,000000000000
5,000000000000
4,000000000000
3,000000000000
2,000000000000
1,000000000000
0,000000000000
1
2
3
4
5
Samplenumber
Error Bars: 95% CI


Multiple Line Mean of uric acid by Samplenumber by BCSK
BCSK
1
2
3
Mean uric acid (mmol/L)
90,0
80,0
70,0
60,0
50,0
40,0
1
2
3
4
5
Samplenumber
Error Bars: 95% CI


Multiple Line Mean of atocopherol by Samplenumber by BCSK
BCSK
1
2
3
Mean alpha-tocopherol (µmol/L)
25,000000000000
20,000000000000
15,000000000000
10,000000000000
5,000000000000
0,000000000000
1
2
3
4
5
Samplenumber
Error Bars: 95% CI


Multiple Line Mean of SODHb by Samplenumber by BCSK
BCSK
1
2
3
Mean SOD (U/g Hb)
50,000000000000
45,000000000000
40,000000000000
35,000000000000
1
2
3
4
5
Samplenumber
Error Bars: 95% CI

Appendix 2: Sample number 3

MDA

BCSK	Mean	N	Std. Deviation
1	,2091	77	,10836
2	,2128	64	,11505
3	,1827	81	,10075
Total	,2005	222	,10803

BCSK		MDA	ALB	FFA	Ceruloplasmin	FRAP	SODHb	BHBZ	GpxHb	URIC	atocopherol
1	Mean	,2091	35,2252	,177	705,38678	232,19779	39,8100239	,65984	4,43159501	65,223	14,3268104
	N	77	77	77	77	77	77	77	77	77	77
	Std. Deviation	,10836	1,88300	,1863	104,765851	41,617514	9,00631397	,195930	1,35080614	10,0301	5,03391349
2	Mean	,2128	35,7045	,155	671,46220	232,74373	44,4185264	,61956	4,66603535	66,417	15,1654262
	N	64	64	64	64	64	64	64	64	64	64
	Std. Deviation	,11505	2,39222	,1115	136,368206	36,236321	12,9628354	,182262	1,30611520	9,3780	5,52911812
3	Mean	,1827	35,6440	,161	701,11972	236,90470	44,5427955	,61431	4,75643733	67,289	14,3530817
	N	81	80	80	81	81	81	80	81	80	81
	Std. Deviation	,10075	2,59475	,1162	130,391077	44,233046	10,0273160	,195551	1,73315981	11,9738	4,74075067
Total	Mean	,2005	35,5156	,165	694,04982	234,07257	42,8654233	,63170	4,61770497	66,317	14,5781590
	N	222	221	221	222	222	222	221	222	221	222
	Std. Deviation	,10803	2,30808	,1431	124,262129	41,022846	10,8247504	,192187	1,49028900	10,5918	5,07003520

Appendix 3: List of abbreviations

BCS: Body condition score
BCSK: body condition score klasse
MDA: malondialdehyde
ROS: reactive oxygen species
SH: thiol groups
SOD: superoxide dismutase
TBARS: thiobarbituric acid-reactive substances
TEAC: trolox equivalent antioxidant capacity
GSH-Px: gluthathione peroxidase
ATP: adenosine-5-triphosphate
FRAP: ferric reducing ability of plasma
NEFA: non-esterified fatty acid
FFA: free fatty acid
BHBA: beta hydroxy butyrate
PUFA: poly unsaturated fatty acid
CP: ceruloplasmin