β-carotene and vitamin E in the dairy industry: blood levels and influencing factors – a case study in Flanders

Beta-caroteen en vitamine E in de melkveehouderij: factoren die bloedconcentraties beïnvloeden – een veldstudie in Vlaanderen

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ABSTRACT

In this case study performed in Flemish dairy herds, it is shown that lactation stage, farm type (grazing (fresh grass) or zero-grazing) and season are interrelated factors associated with circulating β-carotene (bC) and Vitamin E (VitE) concentrations. The iCheck bC is an easy applicable cow-side test to evaluate a cow's bC status. One third of the dairy cows in the study had deficiencies in circulating bC and VitE, especially cows in early lactation and cows from zero-grazing farms. Fresh grass in the diet could not resolve the early post-partum decline in plasma bC and VitE. However, the bC and VitE statuses of dry cows were significantly better on grazing farms. These findings can help updating antioxidant recommendations since it is clear that there is a need for optimization of antioxidant nutritional management in the Flemish dairy industry in order to feed for optimal dairy cow health.

SAMENVATTING

In deze veldstudie uitgevoerd in de Vlaamse melkveehouderij wordt aangetoond dat factoren, zoals lactatiestadium, bedrijfstype (met of zonder weidegang) en seizoen, geassocieerd zijn met plasma-β-caroteen (bC) en vitamine E (VitE)-concentraties bij melkvee. De iCheck bC is een eenvoudig apparaat waarmee de bC-status van een koe snel en op het bedrijf geëvalueerd kan worden. Een derde van de melkkoeien vertoonde deficiënties in plasma-bC en VitE en dit voornamelijk tijdens de vroege lactatie en op bedrijven zonder weidegang. De opname van vers gras kon de daling in plasma-bC en VitE vroeg na de kalving niet opheffen. De bC- en VitE-status van droogstaande koeien was significant beter bij deze met mogelijkheid tot weidegang. Deze bevindingen kunnen bijdragen tot het herformuleren van de diëtaire antioxidant-aanbevelingen met als doel hoogproductieve melkkoeien in optimale gezondheid te houden.

INTRODUCTION

With the onset of lactation, cows enter a period of negative energy balance (NEB) with increased lipolysis resulting in elevated serum non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHB) concentrations (Adewuyi et al., 2005). This rapid mobiliza-tion of body reserves may in turn reduce appetite and thus dry matter intake (Vernon, 2005). Furthermore, this period of increased metabolic demands implies an increase in the production of reactive oxygen species by mitochondria, which are produced as by-products of aerobic metabolism. These changes in oxidative metabolism result in oxidative stress (OS) during

the transition period, and several studies have shown that OS is an incentive for the occurrence of diseases and increases dairy cow susceptibility to suboptimal management, e.g. housing conditions (grazing or stalled), composition of the ration (fresh grass, antioxidant intake) (Bernabucci et al., 2005; Castillo et al., 2005b; Roche, 2006; Sordillo and Aitken, 2009). The total antioxidative capacity of NEB cows is often insufficient (Castillo et al., 2005; De Bie et al., 2014), and may be further reduced by heat stress (Bernabucci et al., 2010) and suboptimal antioxidant uptake through the diet. With fresh grass being the major source of dietary vitamins or antioxidants (AO) such as β-carotene (bC) and vitamin E (VitE) (Ballet et al., 2000), it contributes significantly to the health and antioxidative status of dairy cows. As expanding herd sizes outgrow the 'grazing platform' of a dairy farm, the dairy industry is evolved into zero-grazing systems with increased use of ensiled forages and hay low in vitamins and antioxidants (Reijs et al., 2013; Wilkinson and Rinne, 2018). This, together with the current faster-growing dairy industry and higher-producing animals kept in more intensified dairying, jeopardizes the cow's metabolic health (James, 2012) and might increase the incidence of vitamin and antioxidant deficiencies in the dairy industry. Supplementation guidelines originating from 2001 (NRC) need to be re-evaluated according to the current AO needs in the modern dairy industry (Abuelo et al., 2015). Interventional studies on bC and VitE supplementation are rather univocal confirming that an optimized AO supplementation may positively influence dairy cow health and fertility (Miller et al., 1993; de Ondarza and Engstrom, 2009). Designing ready-to-use AO supplementation protocols is a real challenge due to the lack of a complete understanding of the interrelating factors influencing the AO status of modern high-yielding dairy cows. Moreover, information on the actual bC and VitE statuses of dairy cows (with emphasis on Flanders, the North of Belgium) is lacking, which is valuable information that may contribute to the optimization of the AO status of dairy herds. As such, the authors aimed to: 1) investigate the associations between lactation stage, type of farm (grazing (fresh grass in the diet) or zero-grazing (no fresh grass in the diet)) or season on the one hand and plasma bC and VitE concentrations in dairy cows on the other hand and 2) investigate the current bC and VitE statuses as a measure of the antioxidant status in the dairy industry, using Flanders as a base.

MATERIALS AND METHODS

Selection of dairy farms

Dairy farms in Flanders were invited to participate in a survey to estimate the AO status of high-yielding dairy cows through a call on the website of Dierenge-

zondheidszorg Flanders (DGZ, Drongen, Belgium) in September 2014. Out of 48 interested farms, a total of fourteen were selected, diffusely located in Flanders: seven grazing (presence of fresh grass in the ration) and seven zero-grazing farms (no access to fresh grass). The average Flemish dairy farm in 2015 counted 71 cows and had an annual milk yield per cow of 8,515 kg (Coöperatie Rundvee Verbetering, CRV, 2015). In order to have a representative cohort, only dairy farms with a minimum of fifty lactating animals and a minimum annual milk yield per cow of 8,500 kg were included in the study.

Animals, blood collection and study design

In Figure 1, an overview of the study design is given. All fourteen dairy farms were visited three times: 1) at the beginning of autumn (AUT, Oct-Nov) immediately after the grazing season, 2) at the end of winter (WIN, Feb-Mar) when all cows had been stalled inside for winter and 3) during summer (SUM, Jul-Aug) when cows in grazing farms had access to fresh grass, and day temperatures and temperature humidity indexes (THI) increased (official air temperature and relative humidity at the day of sampling collected from www.meteo.be was used to calculate the THI with http://www.abstechservices.com/?pages=calc4). Each visit, five dry cows (DRY, 2-4 weeks before calving), five cows in early lactation (EARLY LACT, 0-3 weeks after calving) and five cows in mid lactation (MID LACT, at the time of artificial insemination ± 12 weeks after calving) were randomly chosen on each farm. Only multiparous cows were sampled in this study. After disinfecting the skin with 70% ethanol, plasma was sampled from the udder vein in EDTA tubes (BD Vacutainer® K2EDTA, BD, Plymouth, UK) and gently mixed. Serum was sampled in clot activating tubes (BD Vacutainer® SST™ II Avance tubes). After collection, all blood tubes were transported at room temperature and protected from light until further processing.

At the day of blood sampling, the exact number of days after calving, the body condition score (BCS) and parity of each individual cow, and the THI in summer were recorded. In addition, the milk production (mean annual milk yield per cow of each farm) and the average calving interval (CI) of each farm were recorded.

An overview of the composition of the lactation and dry-cow ration (on DM basis) is presented in Table 1. The ration consisted of corn and grass silage, hay, beet pulp, concentrates (and pasture in grazing farms). On grazing-farms, cows were typically allowed on pasture on average 9 hours daily when lactating and 24 hours per day during the dry period. The estimated maximum fresh grass intake of the grazing cows was 6 kg DM/day. During winter, all cows (from grazing- and zero-grazing farms) were stalled inside and did not consume any fresh grass.

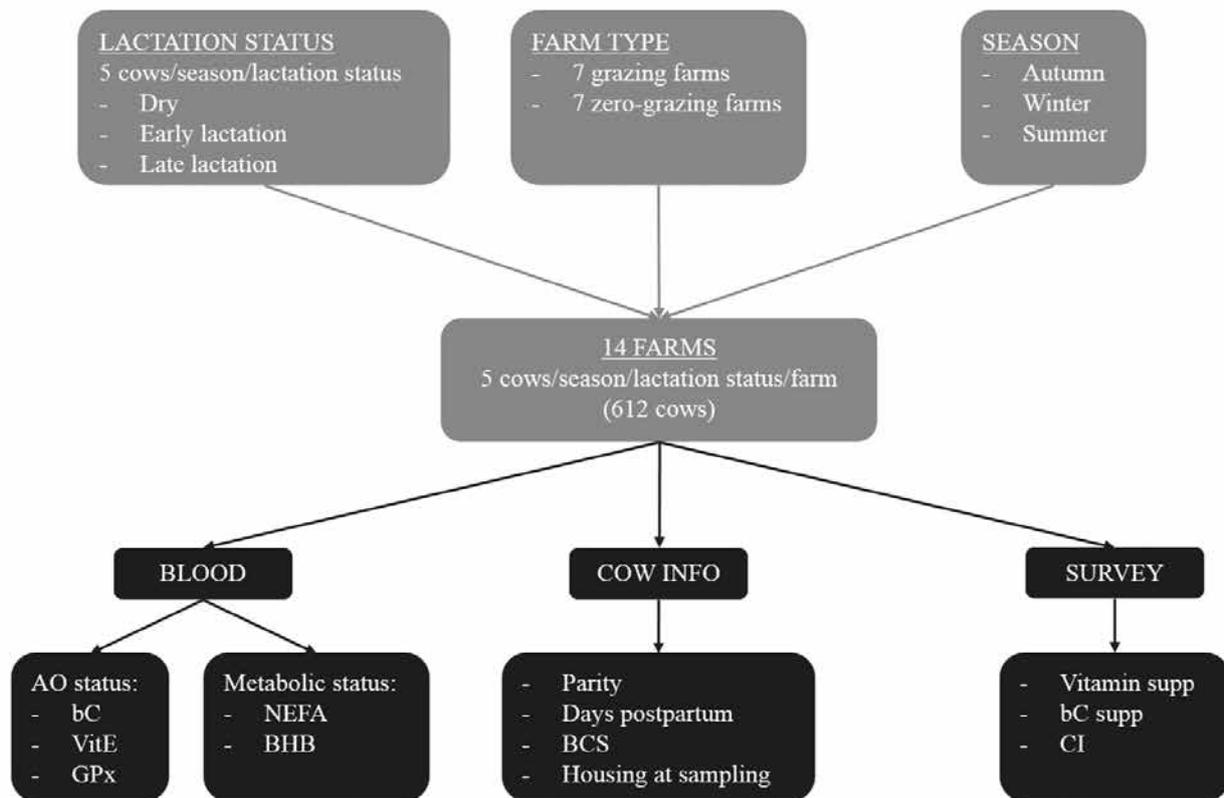The actual intake of fresh grass and other compo-

Figure 1. Study design. Boxes in light grey represent input variables, boxes in dark grey represent output variables. Dry = 2-4 w pre-partum, Early lactation = 0-3 w post-partum, Mid lactation = at time of artificial insemination, bC = β-carotene, VitE = Vitamin E, GPx = Glutathione Peroxidase, NEFA = Non-esterified Fatty Acids, BHB = β-hydroxybutyrate, BCS = Body Condition Score, supp = supplementation, CI = Calving Interval.

Table 1. Detailed composition of the lactation (LACT) and dry (DRY) cow ration in autumn (AUT), winter (WIN) and summer (SUM) in zero-grazing and grazing farms. For the LACT ration, the mean total kg dry matter (DM) of all zero-grazing or grazing farms is presented as well as the proportions (%) of each dietary component calculated on the total DM intake. For the DRY ration, the mean kg DM of each dietary component in zero-grazing and grazing farms is presented. The mean hours that cows were allowed on pasture daily in grazing-farms is presented as well.

	LACT			DRY		
	AUT	WIN	SUM	AUT	WIN	SUM
Zero-grazing farms						
Mean total kg DM/d	20.6 kg	23.3 kg	21.0 kg			
DM corn silage	50.1 %	44.7 %	51.2 %	7.0 kg	7.3 kg	7.5 kg
DM grass silage	19.7 %	23.9 %	20.9 %	3.0 kg	2.6 kg	2.5 kg
DM hay	0.0 %	0.5 %	0.3 %	ad libitum	ad libitum	ad libitum
DM beet pulp	7.7 %	10.8 %	8.0 %	0.7 kg	0.4 kg	0.0 kg
DM concentrates	22.5 %	20.1 %	19.6 %	0.5 kg	0.4 kg	0.7 kg
Grazing farms						
Mean total kg DM/d (excl. fresh grass)	17.3 kg	20.2 kg	15.0 kg			
DM corn silage	44.1 %	44.9 %	46.8 %	5.9 kg	4.4 kg	4.1 kg
DM grass silage	21.7 %	25.6 %	13.9 %	1.5 kg	1.8 kg	2.0 kg
DM hay	0.2 %	1.2 %	0.0 %	ad libitum	ad libitum	ad libitum
DM beet pulp	10.1 %	6.5 %	10.5 %	0.0 kg	0.0 kg	0.0 kg
DM concentrates	23.8 %	21.9 %	28.8 %	0.0 kg	0.0 kg	0.0 kg
Mean hours allowed on pasture/d	8.8	0.0	8.2	24.0	0.0	24.0

Table 2. Linear mixed model building. Significant predictors in the linear mixed models for each of the five outcome parameters (bC, VitE, GPx, logNEFA and logBHB) are presented. No P-values for main effects are shown if the factor lactation stage (LactStage), type of farm (FarmType) or Season was part of a significant interaction (Int) term (although the main effect term itself was kept in the regression model). For each outcome parameter, non-significant factors (P > 0.05; N.S.) were removed from the model, as described in the methods section.

Linear mixed model		bC	VitE	GPx	logNEFA	logBHB
Int	P(LactStage*FarmType)	0.023	0.011	0.0001	N.S.	N.S.
	P(FarmType*Season)	< 0.0001	N.S.	N.S.	N.S.	N.S.
	P(Season*LactStage)	0.002	N.S.	N.S.	N.S.	0.0004
Main effects	P(LactStage)	-	-	-	< 0.0001	-
	P(FarmType)	-	-	-	0.03	N.S.
	P(Season)	-	N.S.	N.S.	< 0.0001	-
	P(bCSupp)	N.S.	N.S.	0.03	N.S.	N.S.
	P(VitSupp)	< 0.0001	< 0.0001	< 0.0001	0.0006	0.001
	P(Parity)	0.04	0.01	N.S.	0.02	0.02
	P(CI)	N.S.	N.S.	N.S.	0.01	N.S.

bC = β-carotene; VitE = Vitamin E; GPx = glutathione peroxidase; NEFA = non-esterified fatty acids, BHB = β-hydroxybutyrate; FarmType = type of farm (zero-grazing or grazing farm); LactStage = lactation stage (dry cows, early in lactation or in mid lactation); Season (autumn, winter or summer); bCSupp = β-carotene supplementation (presence of bC supplement of any kind in the ration); VitSupp = Vitamin supplementation (presence of vitamin supplement of any kind in the ration, including bC); CI = calving interval.

Table 3. Mean plasma β-carotene concentrations (± SEM, µg/mL) split according to FarmType, Season and LactStage. Based on the significant pairwise interactions between lactation stage (LactStage), type of farm (FarmType) and Season in the linear mixed model with β-carotene (bC) as outcome (LactStage*FarmType, FarmType*Season and Season*LactStage; cf Table 2), mean plasma bC ± SEM (µg/mL) is split according to the interacting variables and the effect of the other interacting variable (last column) is reported. The mean plasma bC concentrations and the main effects of LactStage are shown within each Season and FarmType; mean bC and the main effects of FarmType are shown within each Season and LactStage, and mean bC and the main effects of Season are shown within each LactStage and FarmType.

FarmType	Season	LactStage		
		DRY	EARLY LACT	MID LACT
Grazing (2.90 ± 0.09)	AUT (3.63 ± 0.17)	4.40 ± 0.28 [a]	2.48 ± 0.19 [b]	4.04 ± 0.32 [a]
	WIN (2.53 ± 0.12)	2.28 ± 0.16 [a]	2.04 ± 0.20 [a]	3.22 ± 0.21 [b]
	SUM (2.37 ± 0.14)	2.73 ± 0.21 [a]	1.73 ± 0.09 [b]	2.69 ± 0.30 [a]
Zero-grazing (2.54 ± 0.07)	AUT (2.60 ± 0.15)	2.05 ± 0.18 [a]	2.04 ± 0.16 [a]	3.70 ± 0.32 [b]
	WIN (2.74 ± 0.15)	2.21 ± 0.20 [a]	2.36 ± 0.19 [a]	3.64 ± 0.28 [b]
	SUM (2.31 ± 0.08)	2.19 ± 0.12 [a]	1.83 ± 0.11 [a]	2.96 ± 0.15 [b]

LactStage	Season	FarmType	
		Grazing	Zero-Grazing
DRY (2.62 ± 0.10)	AUT (3.21 ± 0.22)	4.40 ± 0.28 [a]	2.05 ± 0.18 [b]
	WIN (2.24 ± 0.13)	2.28 ± 0.16 [a]	2.21 ± 0.20 [a]
	SUM (2.37 ± 0.11)	2.73 ± 0.21 [a]	2.19 ± 0.12 [b]
EARLY LACT (2.09 ± 0.07)	AUT (2.26 ± 0.12)	2.48 ± 0.19 [a]	2.04 ± 0.16 [a]
	WIN (2.20 ± 0.14)	2.04 ± 0.20 [a]	2.36 ± 0.19 [a]
	SUM (1.79 ± 0.08)	1.73 ± 0.09 [a]	1.83 ± 0.11 [a]
MID LACT (3.40 ± 0.11)	AUT (3.87 ± 0.23)	4.04 ± 0.32 [a]	3.70 ± 0.32 [a]
	WIN (3.43 ± 0.18)	3.22 ± 0.21 [a]	3.64 ± 0.28 [a]
	SUM (2.85 ± 0.15)	2.69 ± 0.30 [a]	2.96 ± 0.15 [a]

FarmType	LactStage	Season		
		AUT	WIN	SUM
Grazing (2.90 ± 0.09)	DRY (3.23 ± 0.17)	4.40 ± 0.28 [a]	2.28 ± 0.16 [b]	2.73 ± 0.21 [b]
	EARLY LACT (2.12 ± 0.11)	2.48 ± 0.19 [a]	2.04 ± 0.20 [ab]	1.73 ± 0.09 [b]
	MID LACT (3.37 ± 0.17)	4.04 ± 0.32 [a]	3.22 ± 0.21 [ab]	2.69 ± 0.30 [b]
Zero-grazing (2.54 ± 0.07)	DRY (2.15 ± 0.09)	2.05 ± 0.18 [a]	2.21 ± 0.20 [a]	2.19 ± 0.12 [a]
	EARLY LACT (2.06 ± 0.09)	2.04 ± 0.16 [a]	2.36 ± 0.19 [a]	1.83 ± 0.11 [a]
	MID LACT (3.41 ± 0.15)	3.70 ± 0.32 [a]	3.64 ± 0.28 [a]	2.96 ± 0.15 [a]

DRY = dry period; EARLY LACT = early lactation; MID LACT = mid lactation; WIN = winter; AUT = autumn; SUM = summer; SEM = standard error of the mean. [ab] Data marked with different letters in the same row differ significantly.

nents of the ration can vary significantly under field conditions, as they rely on the appetite of the cow, availability and reachability of food at the feed bunk, competition between animals, etc. Furthermore, the AO content of roughages may vary in time as well, influenced by conservation time, UV (season), pH and others (Ballet et al., 2000). In accordance to LeBlanc et al. (2004), the authors did not study the effects of detailed dietary components and/or exact feed intake in a large multi-herd case study. The specific aim was to broadly screen the average circulating bC and VitE concentrations as a measure of the antioxidative status of grazing (fresh grass in diet) and zero-grazing (no fresh grass in diet) dairy farms. However, next to the presence of fresh grass in grazing farms (pasture or freshly cut grass, regardless of the proportion of this fresh grass in the total ration), the following objectively measurable dietary factors under field conditions were taken into account: 1) whether the cows housed in grazing farms were grazing or had access to fresh grass at the specific moment of blood sampling, 2) whether the farmer added vitamin supplements of any kind in the diet (regardless of the amount) (Yes/No) and 3) whether the farmer added bC supplements to the diet (regardless of the amount) (Yes/No).

Analysis of blood parameters

Inter-assay coefficients of variation (CV) are indicated between brackets. Plasma bC was photometrically analyzed with the iCheck™ (BioAnalyt GmbH, Germany) (2.3 %) according to Schweigert et al. (2007). Serum VitE (9.5 %) was analyzed by means of liquid-liquid extraction and HPLC with UV detection at 292 nm (1260 Infinity, Agilent Technologies, Santa Carla, USA). To estimate the metabolic impact of the negative energy balance in the cows, NEFA (5 %) and BHB (3.5 %) were colorimetrically and enzymatically determined (Randox Laboratories, CrumLin, United Kingdom) in serum with a Gallery™ Plus Automated Photometric Analyzer with detection at 550 nm and 340 nm (Thermo Fisher Scientific, Waltham, USA), respectively. In addition to bC and VitE, plasma concentrations of glutathione peroxidase (GPx; 1.8 % intra-assay, 6.8 % inter-assay CV) were routinely analyzed as a measure of the AO status according to Paglia and Valentine (1967) by means of a commercially available GPx kit (Randox Laboratories, Germany) and were spectrophotometrically detected (Cobas 8000, Rotkreuz, Switzerland).

iCheck β-carotene analysis and evaluation of the antioxidant status of dairy cows

A portable spectrophotometer (iCheck™, Bioanalyt, Germany) was used to assess the blood bC concentrations of each cow. This method was evaluated under field conditions by analyzing identical samples on blood bC with: 1) the portable 'on farm' iCheck™

method (612 cows), 2) a laboratory chromatographic method (liquid-liquid extraction and HPLC with UV-VIS detection at 450 nm, 6.1 % CV, Surveyor LC Pump Plus, Autosampler Plus and PDA Detector, Thermo Fisher Scientific, Belgium) (54 cows) and 3) a laboratory spectrophotometric method (with UV detection at 450 nm, 4.5 % CV, DR3900, Hach Lange, Berlin, Germany) (612 cows). Additionally, blood was also sampled from the tail vein from the same cows (40 cows) in order to evaluate whether the source of blood (udder versus tail vein) influences the iCheck™ bC analysis.

The most recent reference values on optimal serum bC (< 1.5 µg/mL = deficient, 1.5 – 3.5 µg/mL = suboptimal, > 3.5 µg/mL = optimal; Schweigert and Immig, 2007) and VitE concentrations (< 3 µg/mL = deficient, ≥ 3 µg/mL = sufficient; Baldi, 2005) in dairy cows reported in the literature and applied in practice (Calsamiglia and Rodriguez, 2012) were used as benchmark to interpret the antioxidative status of the sampled cows as being optimal, suboptimal or deficient.

Statistical methods

The influence of lactation stage, season and farm type on bC, VitE, GPx, logNEFA, and logBHB (hereafter referred to as 'the outcomes') was modeled using linear mixed models. For each of the five outcomes, the linear mixed model was built using a stepwise backward approach, starting from a full model including lactation stage, season, farm type and their pairwise interactions. In addition, the main effects of parity, bC supplementation, vitamin supplementation and CI were included in the initial model. To account for the dependence between observations in the same cow (random sampling of identical cows occurred occasionally) and for observations within the same farm, a random intercept for cow and farm was included plus random slopes for season and lactation stage. In case of a significant pairwise interaction, the data were split according to one of the interacting variables and the effect of the other interacting variable was reported in the separate groups. If the pairwise interaction was non-significant, the interaction term was removed from the model and the significance of the main effects was tested using the F-test with Kenward-Roger correction for the number of degrees of freedom.

Body condition scores were compared between lactation stages and the annual milk yield per cow of each farm was compared between grazing and zero-grazing farms using a mixed model and one-way ANOVA, respectively. Pairwise correlation between iCheck™ bC and laboratory analyzed bC values or VitE concentrations were expressed using the Pearson correlation coefficient. Plasma bC sampled from the udder vein was compared with plasma bC withdrawn from the tail vein using one-way ANOVA. A log trans-

Table 4. Mean plasma vitamin E concentrations (± SEM, µg/mL) split according to FarmType and LactStage. Based on the significant pairwise interaction between lactation stage (LactStage) and type of farm (FarmType) in the linear mixed model with vitamin E (VitE) as outcome (LactStage*FarmType; cf Table 2), mean plasma VitE ± SEM (µg/mL) is split according to the interacting variables and the effect of the other interacting variable (last column) is reported. The mean plasma VitE concentrations and the main effects of LactStage are shown within each FarmType; mean VitE and the main effects of FarmType are shown within each LactStage.

FarmType	LactStage		
	DRY	EARLY LACT	MID LACT
Grazing (4.10 ± 0.10)	4.50 ± 0.20 [a]	2.90 ± 0.20 [b]	5.00 ± 0.20 [a]
Zero-grazing (3.90 ± 0.10)	3.40 ± 0.10 [a]	3.00 ± 0.10 [a]	5.30 ± 0.20 [b]
LactStage	**FarmType**		
	Grazing	Zero-grazing	
DRY (3.90 ± 0.10)	4.50 ± 0.20 [a]	3.40 ± 0.10 [b]	
EARLY LACT (3.00 ± 0.10)	2.90 ± 0.20 [a]	3.00 ± 0.10 [a]	
MID LACT (5.20 ± 0.10)	5.00 ± 0.20 [a]	5.30 ± 0.20 [a]	

DRY = dry period; EARLY LACT = early lactation; MID LACT = mid lactation; SEM = standard error of the mean. [ab] Data marked with different letters in the same row differ significantly.

Table 5. Mean red blood cell glutathione peroxidase concentrations (± SEM, U/gHb) split according to FarmType and LactStage. Based on the significant pairwise interaction between lactation stage (LactStage) and type of farm (FarmType) in the linear mixed model with glutathione peroxidase (GPx) as outcome (LactStage*FarmType; cf Table 2), mean red blood cell GPx ± SEM (U/gHb) is split according to the interacting variables and the effect of the other interacting variable (last column) was reported. The mean plasma GPx concentrations and the main effects of LactStage are shown within each FarmType; mean GPx and the main effects of FarmType are shown within each LactStage.

FarmType	LactStage		
	DRY	EARLY LACT	MID LACT
Grazing (510.90 ± 12.20)	556.70 ± 25.00 [a]	471.40 ± 19.20 [b]	509.40 ± 18.70 [b]
Zero-grazing (499.90 ± 10.40)	480.90 ± 18.80 [a]	488.5 ± 16.50 [a]	530.40 ± 19.20 [b]
LactStage	**FarmType**		
	Grazing	Zero-grazing	
DRY (512.40 ± 15.30)	556.70 ± 25.00 [a]	480.90 ± 18.80 [a]	
EARLY LACT (480.90 ± 12.50)	471.40 ± 19.20 [a]	488.50 ± 16.50 [a]	
MID LACT (521.1 ± 13.5)	509.40 ± 18.70 [a]	530.40 ± 19.20 [a]	

DRY = dry period; EARLY LACT = early lactation; MID LACT = mid lactation; SEM = standard error of the mean. [ab] Data marked with different letters in the same row differ significantly.

Table 6. Mean plasma non-esterified fatty acid concentrations (± SEM, mM) in each LactStage, FarmType and Season. Based on the non-significant pairwise interactions between lactation stage (LactStage), type of farm (FarmType) and Season in the linear mixed model with non-esterified fatty acids (NEFAs) as outcome (LactStage*FarmType, FarmType*Season and Season*LactStage; cf Table 2), mean serum NEFA ± SEM (mM) and the main effects of LactStage, FarmType and Season are presented.

LactStage		
DRY	EARLY LACT	MID LACT
0.24 ± 0.02 [a]	0.36 ± 0.02 [b]	0.14 ± 0.01 [c]
FarmType		
Grazing	Zero-grazing	
0.24 ± 0.02 [a]	0.25 ± 0.01 [b]	
Season		
AUT	WIN	SUM
0.18 ± 0.02 [a]	0.22 ± 0.02 [a]	0.34 ± 0.02 [b]

DRY = dry period; EARLY LACT = early lactation; MID LACT = mid lactation; WIN = winter; AUT = autumn; SUM = summer; SEM = standard error of the mean. [abc] Data marked with different letters in the same row differ significantly.

Table 7. Mean plasma β-hydroxybutyrate concentrations (± SEM, mM) split according to Season and LactStage. Based on the significant pairwise interaction between lactation stage (LactStage) and Season in the linear mixed model with β-hydroxybutyrate (BHB) as outcome (Season*LactStage; see Table 2), mean serum BHB ± SEM (mM) is split according to the interacting variables and the effect of the other interacting variable (last column) was reported. The mean serum BHB concentrations and the main effects of LactStage are shown within each season, and mean serum BHB concentrations and the main effects of season are shown within each LactStage.

Season	LactStage		
	DRY	EARLY LACT	MID LACT
AUT (0.68 ± 0.04)	0.80 ± 0.10 [a]	0.63 ± 0.04 [a]	0.61 ± 0.06 [a]
WIN (0.70 ± 0.04)	0.66 ± 0.05 [ab]	0.91 ± 0.09 [a]	0.52 ± 0.03 [b]
SUM (0.80 ± 0.05)	0.74 ± 0.06 [ab]	1.10 ± 0.13 [a]	0.59 ± 0.03 [b]

LactStage	Season		
	AUT	WIN	SUM
DRY (0.73 ± 0.04)	0.80 ± 0.10 [a]	0.66 ± 0.05 [a]	0.74 ± 0.06 [a]
EARLY LACT (0.88 ± 0.05)	0.63 ± 0.04 [a]	0.91 ± 0.09 [ab]	1.10 ± 0.13 [b]
MID LACT (0.57 ± 0.02)	0.61 ± 0.06 [a]	0.52 ± 0.03 [a]	0.59 ± 0.03 [a]

WIN = winter; AUT = autumn; SUM = summer; DRY = dry period; EARLY LACT = early lactation; MID LACT = mid lactation; SEM = standard error of the mean. [ab] Data marked with different letters in the same row differ significantly.

Table 8. Details of cows in each lactation stage. The total number of cows in each lactation stage (LactStage) is presented as well as the mean days post-partum, mean parity and mean body condition score (BCS).

Item	LactStage		
	DRY	EARLY LACT	MID LACT
Total number of cows	198	206	208
Mean days post-partum	392.4 ± 6.5	17.8 ± 0.9	79.8 ± 2.2
Mean parity	2.3 ± 0.1	3.0 ± 0.1	2.7 ± 0.1
Mean BCS	3.27 ± 0.04 [a]	2.75 ± 0.04 [b]	2.57 ± 0.03 [c]

Statistics were only performed on BCS. [abc]Data marked with different letters in the same row differ significantly.

formation was applied to correct for abnormality and inhomogeneity of variances when necessary. All data were presented as means ± SEM. Analyses were carried out in IBM SPSS Statistics 23 for Windows (Chicago, IL, USA) or in R 3.2.1 (R Core Team, 2014). The threshold for statistical significance was set at P < 0.05.

RESULTS

The original data of this study are available at Mendeley Data (http://dx.doi.org/10.17632/vzn5s-5mtty.1).

To model the effect of lactation stage, season and farm type on bC, VitE, GPx, logNEFA, and logBHB, linear mixed models were fitted. The significant terms from these models for each outcome parameter are shown in Table 2. Means ± SEM are shown in Tables 3 to 7 including the main effects of interacting variables, taking into account the other interacting variables. Effect sizes accounting for significant effects of parity, bC supplementation, vitamin supplementation and CI are described below.

Animals

A total of 630 cows from fourteen farms situated in Flanders were sampled, of which 612 samples were successfully analyzed. The total number of sampled cows in each lactation stage (DRY, EARLY LACT and MID LACT) as well as mean days post-partum, parity and BCS are presented in Table 8. The body condition scores were significantly different with the lowest scores in MID LACT and the highest scores in DRY cows (P < 0.01). The dairy farms included in this study counted a mean of 106 ± 12 cows (84 ± 7 in grazing farms, 128 ± 19 in zero-grazing farms) and had an average annual milk yield per cow of 9,280 ± 188 kg. The mean annual milk yield per cow on each farm did not significantly differ between grazing (9,166 ± 256 kg) and zero-grazing farms (9,394 ± 290 kg) and was therefore not further taken into account in the final statistical model.

β-carotene

The final model for bC included significant interactions between lactation stage (LactStage), type of

farm (FarmType) and season, as well as significant main effects for vitamin supplementation (VitSupp) and parity (cf Table 2 for exact P-values). Since there are no main effects of LactStage, FarmType and Season on plasma bC, the effect of LactStage is separately reported by Season and FarmType in Table 3. In each FarmType and season, EARLY LACT was associated with a significantly lower plasma bC compared with MID LACT. In grazing farms in AUT and SUM, the DRY period was associated with significant higher bC concentrations in cows than in EARLY LACT cows, which was not the case in cows from zero-grazing farms. Similarly, when focusing on the effect of Farm-Type in each Season and LactStage (Table 3), only in DRY cows in AUT and SUM, bC concentrations were significantly higher in cows from grazing farms than in cows from zero-grazing farms. Additionally, when focusing on the effect of Season in each LactStage and FarmType (Table 3), bC concentrations in cows from grazing farms were significantly lower in SUM than in AUT. Interestingly, this reduction in circulating bC during SUM was not present in cows housed under zero-grazing conditions.

VitSupp (but not bCSupp) was associated with increased plasma bC in DRY cows from grazing farms in all seasons ($+1.25 \pm 0.28$ µg/mL bC). In contrast, VitSupp in MID LACT was significantly linked to reduced plasma bC concentrations (-1.27 ± 0.49 µg/mL bC in grazing farms and -1.04 ± 0.49 µg/mL bC in zero-grazing farms). Also parity was a factor associated with plasma bC, with a slightly reduced plasma bC concentration with increased parity (-0.11 ± 0.05 to -0.31 ± 0.10 µg/mL bC depending on FarmType, LactStage and season).

Vitamin E

Whereas season did not alter VitE concentrations, LactStage and FarmType significantly interacted and influenced plasma VitE concentrations (cf Table 2 for exact P-values). Similarly to bC, VitSupp and Parity significantly altered plasma VitE concentrations and were included as a dependent variable in the final model with VitE as outcome. The main effects of LactStage and FarmType on plasma VitE could not be calculated separately and thus the effect of LactStage in each FarmType was investigated and is shown in Table 4. The effects of LactStage and FarmType on VitE concentrations were similar to the effects observed on circulating bC. VitE concentrations were significantly lower in EARLY LACT than in MID LACT cows in both types of farms. In grazing farms, DRY cows had significant higher plasma VitE than EARLY LACT cows. Similarly, when focusing on the effect of FarmType in each LactStage (Table 4), VitE concentrations were significantly higher in DRY cows in grazing farms than in zero-grazing farms.

VitSupp (but not bCSupp) was associated with increased plasma VitE concentrations in both farm types, but only in DRY cows ($+1.00 \pm 0.28$ µg/mL VitE). Increasing parity was linked to reduced plasma VitE concentrations (-0.18 ± 0.06 µg/mL VitE), but only in cows on grazing farms.

Glutathione peroxidase

LactStage and FarmType significantly interacted and affected plasma GPx concentrations (cf Table 2 for exact P-values). Season did not influence plasma GPx concentrations. VitSupp and bCSupp but not Parity significantly altered plasma GPx concentrations as well, and were included as a dependent variable in the final model with GPx as outcome. The main effects of LactStage and FarmType on plasma GPx could not be calculated separately and thus the effect of LactStage in each FarmType was investigated (Table 5). Only in cows from zero-grazing farms, GPx concentrations were significantly lower in EARLY LACT than in MID LACT. DRY cows from grazing farms had significantly higher plasma GPx concentrations than MID LACT cows and EARLY LACT cows. Regardless of these findings, no significant impact of type of farm was found (Table 5).

VitSupp and bCSupp were significantly associated with reduced plasma GPx concentrations, but only in cows from grazing farms (-80.76 ± 27.29 U/gHb GPx when supplemented with vitamins and -159.02 ± 46.36 U/gHb GPx when supplemented with bC).

Blood parameters for energy balance

Non-esterified fatty acids

Plasma NEFA concentrations were heavily skewed and subsequently log-transformed in the final model. None of the three main factors (LactStage, FarmType and Season) significantly interacted for logNEFA concentrations, but they all had a significant main effect on plasma logNEFA concentrations (cf Table 2 for exact P-values). VitSupp, parity and CI were significantly linked to plasma logNEFA and are included as a dependent variable in the final model with logNEFA as outcome. Mean NEFA concentrations ± SEM in each group of interest are shown in Table 6. Circulating NEFAs were significantly higher in EAR-LY LACT cows than in DRY and MID LACT cows (Table 6). Plasma NEFA concentrations were significantly, but only 0.01 mM higher in cows from zero-grazing farms than in cows from grazing farms (Table 6). Plasma NEFA were significantly higher during SUM than during AUT and WIN (Table 6). VitSupp was linked to reduced plasma NEFA concentrations (-0.04 ± 0.06 mM NEFA), whereas increasing parity and CI were associated with increased circulating NEFAs ($+0.03 \pm 0.01$ and $+0.01 \pm 0.00$ mM NEFA, respectively).

B-hydroxybutyrate

Plasma BHB concentrations were heavily skewed
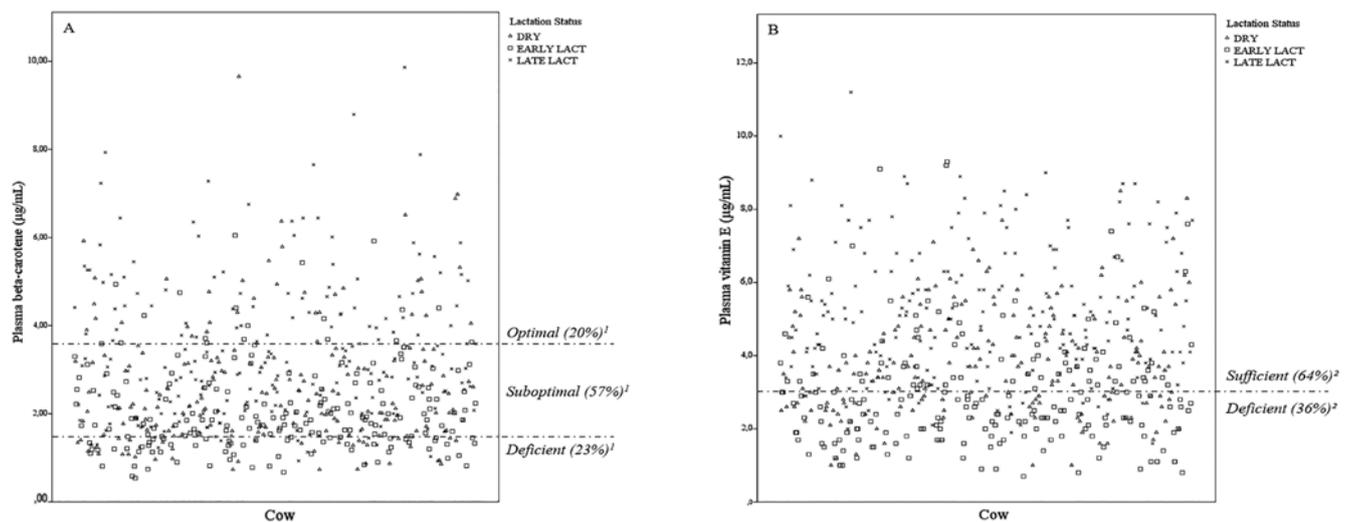
Figures 2A. Beta-carotene and 2B. vitamin E statuses of dairy cows under field conditions. Beta-carotene (analyzed by means of the iCheck™) and vitamin E values of each individual cow are presented according to lactation stage (DRY = dry cows, 2 to 4 weeks before calving; EARLY LACT = early lactation cows from 0 to 3 weeks after calving; MID LACT = mid lactation cows at the moment of artificial insemination). [1]Reference values for beta-carotene are based on Schweigert and Immig (2007): < 1.5 μg/mL = deficient, 1.5 – 3.5 μg/mL = suboptimal, > 3.5 μg/mL = optimal. [2]Reference values for vitamin E are based on Baldi (2005): < 3 μg/mL = deficient, > 3 μg/mL = sufficient. Percentages of cows in each category are indicated between brackets.

and subsequently log-transformed in the final model. Season and LactStage significantly interacted and affected plasma logBHB concentrations (cf Table 2 for exact P-values). FarmType did not influence plasma logBHB concentrations. VitSupp and parity were significantly associated with plasma logBHB concentrations and were included as a dependent variable in the final model with logBHB as outcome. Mean BHB concentrations ± SEM dependent on LactStage and Season are shown in Table 7. Only in WIN and SUM, EARLY LACT cows had significantly higher plasma BHB concentrations than MID LACT cows. In EARLY LACT cows, BHB concentrations were significantly increased in SUM than in AUT. Regardless of a significant main effect of VitSupp and Parity on BHB concentrations (Table 2), no differences were found in plasma BHB in cows due to VitSupp or Parity.

iCheck β–carotene analysis and the antioxidant status of dairy cows

The iCheck™ bC concentrations measured under field conditions (n = 612) correlated significantly with the spectrophotometric bC concentrations (n = 612) (R = 0.798) and the chromatographic bC concentrations (n = 54) (R = 0.769) measured under laboratory conditions. β-carotene in blood sampled from the udder vein (3.55 ± 0.24 μg/mL, n = 40) was similar to blood bC concentrations withdrawn from the tail vein (3.63 ± 0.25 μg/mL, n = 40). ICheck bC also correlated significantly with VitE concentrations (R = 0.668). The results of the bC and VitE statuses of Flemish dairy cows are presented in Figures 2 a and 2b. When bC reference values were applied, 20 % of the sam-

pled cows had an optimal bC level of > 3.5 μg/mL, whereas 23 % of the cows had deficient bC concentrations < 1.5 μg/mL. The majority of cows had suboptimal levels of bC (between 1.5 and 3.5 μg/mL). In case of VitE, 64 % of the cows had sufficient levels of VitE (≥ 3 μg/mL), but 36 % of the sampled cows showed deficient circulating VitE concentrations (< 3 μg/mL). Notably, the majority of cows with deficient bC and VitE levels were EARLY LACT cows (54 % for bC and 59 % for VitE), while DRY cows accounted for 34 % (bC) and 30 % (VitE) and MID LACT cows for 12 % (bC) and 11 % (VitE). In total, 77 % of the cows deficient in bC and 59 % of the cows with deficient VitE levels were cows from zero-grazing farms.

DISCUSSION

β-carotene and VitE have been proposed as important antioxidants in metabolically stressed dairy cows, as their serum concentrations are indicative for their antioxidative status and linked to health and fertility outcomes (Jukola et al., 1996; Ayasan and Karakozak, 2010; Nayyar and Jindal, 2010). However, knowledge of the actual bC and VitE statuses in dairy cows as well as the interrelationship of factors influencing that antioxidant status is currently lacking. In this study, the authors aimed to investigate the antioxidant status (focusing on bC and VitE) in dairy farms in Flanders taking into account factors, such as lactation stage of the cow, type of farm (grazing or zero-grazing) and season. In the study, it was shown that these factors were associated with the levels of plasma bC and VitE and red blood cell GPx and that they were interrelated. One third of the dairy cows sampled in this study

had deficient circulating bC and VitE concentrations. It could be confirmed that especially the early post-partum period is the most critical one in terms of circulating bC, VitE and GPx, coinciding with the NEB status.

Plasma bC and VitE levels reached their nadir early post-partum, whereas serum NEFA and BHB were highest during this period, indicative for a metabolic status of NEB (Drackley et al., 2001). This reduction in bC and VitE concentrations peri-partum has been reported previously (Calderon et al., 2007; Sharma et al., 2011) and can be attributed to: 1) the reduced dry matter intake post-partum (Grummer et al., 2004; Calderon et al., 2007) resulting in less antioxidant uptake from the ration, 2) the increased use of antioxidants during this NEB state since cows suffer from elevated OS early post-partum (Bernabucci et al., 2002) or 3) the massive increased milk production and loss of fat soluble antioxidants via colostrum and milk (Calderon et al., 2007; Kankofer and Albera, 2008).

The type of farm is associated with altered plasma bC and VitE concentrations in dry cows only. This implies that fresh grass-based diets high in bC and VitE can increase plasma bC and VitE levels in dry cows, as shown by Calderon et al. (2007). However, dry cows at grazing farms are often kept on fields with low quality pasture to avoid high calcium and potassium intakes pre-partum. As such, the observed higher bC and VitE levels in dry cows from grazing-farms may be explained by the release of bC and VitE reserves from fat depots when needed, which will not be excreted in dry cows via milk. This accumulation in lipid stores most probably takes place during the last phase of lactation when cows are in positive energy balance and still grazing on pasture (Baldi, 2005; Noziere et al., 2006). Similar to the observed increase in AO in dry cows under grazing conditions, the authors previously showed that bC supplementation exceeding daily recommendations (>300 – 1.000 mg bC per head per day (Calsamiglia and Rodriguez, 2012), but matching the amount of bC intake at grazing (2.000 mg bC; Kawashima et al., 2010) in non-lactating (dry, but not pregnant) cows, could increase circulating bC levels (De Bie et al., 2016). Moreover, this interventional study also showed that daily bC supplementation was associated with increased bC levels in NEB cows as well (De Bie et al., 2016). In the present field study, grazing could not be linked with increased bC levels in NEB cows in early lactation. In accordance, Johansson et al. (2014) showed that cows in organic dairy farms, receiving fresh grass and legume silages, could fulfill their VitE and VitA (metabolite of bC) requirements without supplementation, except at the time around peak lactation. These observations further emphasize the impact of the three factors described above (reduced DMI and thus AO uptake, increased OS, increased loss of AO via milk) on the cow's AO status early post-partum, explaining the absence of any effect of fresh grass in the diet on bC levels in lactating cows.

Season was significantly associated with altered plasma bC concentrations, but only in cows from grazing farms. Unexpectedly, bC levels in cows from grazing farms were not highest during SUM when all cows were grazing, but in AUT when 52% of the cows had already been stalled and temperature had cooled down. In four out of five grazing farms in SUM, blood was collected at a moment when the THI reached a mean of 79 ± 4, indicating moderate stress due to heat (Armstrong, 1994). Heat stress may be responsible for the reduced circulating bC concentrations observed in SUM (Quintela et al., 2008). In addition, fresh grass contains high concentrations of bC, which is sensitive to breakdown by UV, lowering the bC uptake from the ration and thus resulting in reduced circulating bC in SUM (Ballet et al., 2000). In zero-grazing farms, no effect of season on circulating bC concentrations could be observed. This may be attributed to the presence of shade in the stables, which is known to reduce heat stress (Armstrong, 1994), or to the fact that no fresh grass was present in the ration of cows housed in zero-grazing farms that is vulnerable to breakdown by UV.

Next to dietary antioxidants, GPx (present in e.g. erythrocytes) is an important intracellular enzyme, which represents a major antioxidant defence mechanism in the body (Cohen and Hochstein, 1963). In the present study, a decline in red blood cell GPx was observed during early lactation, which is consistent with other reports (Festila et al., 2013; Konvičná et al., 2015). The highest GPx concentrations were observed in dry cows from grazing farms. It can be assumed that the availability of fresh grass during or prior to the dry period can increase the capacity of defence mechanisms against OS, which can be emphasized by the earlier described high bC and VitE levels in dry cows from grazing farms. As such, the type of ration prior to or during the dry period seems to be of high importance for the circulating bC, VitE and GPx levels of dry cows in particular. In this regard, most recent supplementation guidelines recommend higher supplementation of bC and VitE in cows during the dry and early lactation period than in late post-partum cows (Calsamiglia and Rodríguez, 2012).

As described above, bC and VitE levels in dairy cows vary according to lactation stage, farm type and season, but type of forage or nutritional vitamin uptake is the predominant factor affecting circulating bC and VitE (Noziere et al., 2006). As such, the influence of vitamin and bC supplements was taken into account in this study. Increased plasma bC and VitE levels were detected when the farmer indicated that extra vitamins were supplemented in the ration, but only in dry cows. Surprisingly and in contrast, vitamin and/or bC supplementation in mid lactating cows was associated with reduced plasma bC and reduced red blood cell GPx concentrations. Caution needs to be taken when interpreting these results, especially

because farmers with herd problems may supplement their cows more easily in order to improve dairy cow health, transition, production and/or reproduction. As such, the factor 'vitamin or bC supplementation' may be associated with reduced circulating AO as a result of health problems during the transition period that are associated with increased oxidative stress and thus reduced circulating AO.

In search for the bC status of cows, an easy applicable cow side test (iCheck™) was used. This portable spectrophotometer allows direct on-farm analysis of whole blood samples from the tail vein within five minutes. Schweigert and Immig (2007) have validated this cow side bC test under controlled experimental conditions and showed high correlations between iCheck bC and bC analyzed by means of HPLC (R=0.990). For the first time, this bC test has been used under variable field conditions and showed good correlations with bC analyzed by HPLC (R=0.798). In this way, the dairy farmer and the veterinarian are provided with a highly efficient analyzing strategy to rapidly screen the bC status of its herd (Schweigert and Immig, 2007). Moreover, thanks to the correlation between iCheck bC and VitE, the VitE status can also be estimated based on the iCheck bC levels. These blood bC and VitE concentrations contribute to the AO defence system (Sies, 1993), which provides an indication of the AO status of cows within a farm. Results of this screening in the present study showed that about one fourth of all cows were deficient in circulating bC levels while one third of the cows had deficient VitE levels. Not surprisingly, the majority of the cows deficient in bC and VitE were cows in early lactation and on zero-grazing farms. Nevertheless, still a significant number of cows on grazing farms (± one third) had deficient bC and VitE concentrations. As previously discussed, it is clear that the early lactation stage, in which cows suffer from a NEB period is responsible for these deficiencies in bC and VitE both on grazing as well as on zero-grazing farms. Focusing on reproduction, this NEB induced reduction in AO levels is reflected in the oocyte's micro-environment or follicular fluid. Strategically supplied bC can improve antioxidant concentrations in the follicular fluid regardless of the energy state of the cow, which provides opportunities for improvement of fertility (De Bie et al., 2016). However, that study was performed in a non-lactating dietary induced NEB cow model. Integrating this with the data of the present study, it can be concluded that optimizing the AO status in early lactating dairy cows may be a challenge. Nowadays, commercially established vitamin requirements have substantially increased (Calsamiglia and Rodríguez, 2012), with an obvious need for increases in daily bC and VitE supplementations per head. Despite the existence of these commercial advices and the awareness of the importance of optimal vitamin nutrition by dairy farmers (reflected by the fact that 71% of the Flemish dairy farms in this study supplemented

their cows with vitamins of any kind), still 25 to 35% of the Flemish dairy cows have deficient bC and VitE levels. Possibly, the lack of knowledge on the factors influencing these circulating antioxidants and thus the ignorance of when to supplement and which cows to supplement might be responsible for the deficiencies observed in the dairy industry. Moreover, in 2001, the NRC concluded that there was insufficient knowledge on the bC action in dairy cattle and the factors influencing its presence to be able to formulate any official requirements for dairy cows (NRC, 2001). The knowledge generated in the present study may help to formulate new bC and VitE recommendations, since it is clear that there is a need for the optimization of antioxidant nutritional management in the dairy industry, especially in early lactating cows and cows from zero-grazing farms.

The farms included in this case study are representative for the average dairy farm in Flanders based on average milk yield and mean number of cows per farm. Models predict that the number of grazing farms in North-West Europe will keep on declining from two thirds of the cows currently grazing to only one third grazing in 2025 (Reijs et al., 2013). These results possibly also apply to the Southern part of Belgium and other countries throughout Europe and beyond which have a similar climate and follow the same trend of a reduced number of grazing farms and an increased use of silages. This furthermore emphasizes the growing importance of the optimization of AO nutrition in the dairy industry in general. The authors believe that the conclusions drawn in this study are interesting for and may be applicable to dairy farms in Western Europe managed in similar climate and feeding conditions as seen in the cohort of the current study.

CONCLUSION

The dataset revealed that the factors influencing circulating bC and VitE are all interrelated and mainly depend on the metabolic state of the cow and management practices of the farmer: lactation stage of the cow, farm type (grazing and zero-grazing farms), season, extra vitamin supplementation and parity. The presence of fresh grass in the ration could not prevent the NEB induced decline in circulating bC, VitE and GPx, but it seems that dry cows in particular benefit most from being housed on a grazing farm. Finally, at least one third of dairy cows was shown to have deficient circulating bC and VitE concentrations, especially cows in early lactation and cows housed under zero-grazing conditions. The easily applicable cow side bC test (iCheck™) is used under field conditions and is ideal to easily assess a cow's bC status. In this study, it is emphasized that more attention on antioxidant nutrition in the dairy industry is required, which would provide an important opportunity for improvement of health and fertility of the modern high yield-

ing dairy cow kept under rapidly evolving management conditions.

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