α-Tocopherol and β-Carotene in Forages and their Utilisation by Dairy Cows in Organic Production

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Cover: Birdsfoot trefoil and timothy mixture
(photo: Annika Arnesson)

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Abstract

There are large variations in α-tocopherol (vitamin E) and β-carotene (pro-vitamin A) concentrations in forages. α-Tocopherol and β-carotene are antioxidants and important for the health and fertility of cows. This thesis investigated the effects of site, year, forage species, cutting date, wilting and ensiling technique on α-tocopherol and β-carotene concentrations in forage. Utilisation of vitamins E and A in dairy cows was also studied. Different grass-legume mixtures of the forage species birdsfoot trefoil, red clover, timothy and meadow fescue were sown at two sites in Sweden; Skara in the south and Umeå in the north. The mixtures were harvested at three occasions in the spring growth. At an additional site in Denmark, perennial ryegrass was used instead of meadow fescue. White clover was also included in Denmark and forages were harvested in a four-cut system. Vitamin analysis was performed on forage species from all mixtures and cutting dates. Silages treated with acid or bacterial inoculant were compared to untreated silages from a regrowth of forage mixtures in Skara. Dairy cows were offered a high daily dose of esterified natural vitamin E during the transition period (E) and were compared to cows without supplementation (C). Concentrations of α-tocopherol, β-carotene and retinol were measured in the blood and milk during lactation. Forages grown at Skara generally had higher vitamin concentrations than forages grown at Umeå. Differences between years were smaller. Birdsfoot trefoil generally had higher vitamin concentrations than red clover, whereas differences between grasses were more inconsistent. Cutting date had less effect at Umeå than Skara, irrespective of leaf blade proportion and developmental stage. In Denmark, numerical differences indicate that vitamin concentrations were higher in the fourth harvest than in earlier harvests, which was possibly a seasonal effect. The α-tocopherol concentration in the mixture with birdsfoot trefoil increased during ensiling and was higher in silages with birdsfoot trefoil than in silages with red clover. Inoculant-treated silage with red clover had higher α-tocopherol content than untreated silage with red clover. The E cows had higher concentrations of α-tocopherol in the blood than the C cows, when the supplement was offered. No effects of treatment on vitamin concentrations in blood were found later in the lactation. Treatment effects on vitamin concentrations in milk were affected by the vitamin concentration of the forage.

Keywords: α-tocopherol, β-carotene, grass, legume, forage, silage, organic, dairy cow

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Dedication

To all my loving friends and family...

Man ska inte påpeka sånt som inte syns.
    Lisa Sellberg

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Lindqvist, H., Gustavsson, A.-M., Jensen, S.K., Nilsdotter-Linde, N. & Nadeau, E. (2012). α-Tocopherol and β-carotene in forage legumes and grasses during spring growth – effects of site, year, species and cutting date. (manuscript).

II Lindqvist, H., Nadeau, E., Jensen, S.K. & Søegaard, K. (2012). α-Tocopherol and β-carotene contents of forage species in a four-cut system. (submitted).

III Lindqvist, H., Nadeau, E. & Jensen, S.K. (2012). α-Tocopherol and β-carotene in legume-grass mixtures as influenced by wilting, ensiling and type of silage additive. *Grass and Forage Science* 67, 119-128.

IV Lindqvist, H., Nadeau, E. Persson-Waller, K., Jensen, S.K. & Johansson B. (2011). Effects of RRR-α-tocopheryl acetate supplementation during the transition period on vitamin status in blood and milk of organic dairy cows during lactation. *Livestock Science* 142, 155-163.

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Abbreviations

AI   Artificial insemination
BCS   Body condition score
BEC   Before expected calving
BFT   Birdsfoot trefoil
BW   Body weight
C   Control treatment
CP   Crude protein
CNCPS   Cornell Net Carbohydrate and Protein System
DM   Dry matter
E   Supplemented with semi-natural vitamin E
EXP   Experiment
GGDP   Geranylgeranyl diphosphate
IPP   Isopentyl diphosphate
IU   International units
MEP   2-C-methyl-D-erytritol-4-phosphate
MF   Meadow fescue
MPBQ   Methyl-6-phytyl-1,4-benoquinone
NDF   Neutral detergent fibre
PC   Post calving
PDP   Phytyl-diphosphate
PR   Perennial ryegrass
RC   Red clover
ROS   Reactive oxygen species
SCC   Somatic cell count
TI   Timothy
WC   White clover
α-TTP   α-Tocopherol transfer protein

1 Introduction

Vitamins are defined as a group of essential complex organic compounds present in small amounts in natural feed and foodstuffs (McDowell, 2000). They are divided into fat-soluble or water-soluble vitamins. Fat-soluble vitamins include A, D, E and K, while vitamins B and C are classified as water-soluble (McDowell, 2000).

Dairy cows usually fulfil their requirements of vitamins B, C and K without dietary supplementation, as the micro-organisms in the rumen are believed to synthesis sufficient amounts (McDowell, 2000). Vitamin D requirements could be fulfilled by endogenous production from summer sunlight when the dairy cows are out on pasture (Hymøller *et al.*, 2009). However, dietary vitamin D supplementation is recommended, at least for the indoor period (NRC, 2001; Hymoller *et al.*, 2009).

Requirements of vitamins A and E for dairy cows may be difficult to fulfil only by natural vitamins in the basal diets (NRC, 2001). The requirements are especially high around calving due to increased secretion of vitamins into the udder, decreased dry matter intake and increased physiological stress (Goff & Stabel, 1990; Drackley, 1999; NRC, 2001).

Both vitamins A and E are usually supplemented synthetically (Herdt & Stowe, 1991). However, several studies have reported benefits of supplementing with at least vitamin E in a natural form or the esterified (semi-natural) form (Meglia *et al.*, 2006; Weiss *et al.*, 2009).

The studies in this thesis examined the possibilities to increase the natural concentrations of α-tocopherol (vitamin E) and β-carotene (pro-vitamin A) in the diet of dairy cattle. As forage is the largest source of natural α-tocopherol and β-carotene in dairy production, the studies centred on how to increase the vitamin content in forage through choice of forage species, cutting date and conservation method. One study also examined supplementation of semi-natural vitamin E to dairy cows in organic production.

1.1 Nomenclature and function

1.1.1 Nomenclature of α-tocopherol

Vitamin E refers to a series of compounds, called tocopherols and tocotrienols. They can be synthesised by plants and other oxygenic photosynthetic organisms (DellaPenna, 2005). Eight forms of vitamin E exist: four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ). They differ in the number and position of methyl substitutes on the chromanol ring system with α having three methyl substitutes, β and γ having two and δ one (Figure 1). Tocopherols and tocotrienols differ in the degree of saturation of their hydrophobic tails (DellaPenna, 2005; Havaux *et al.*, 2005).

The thesis focus on α-tocopherol as it has the highest biological activity and is the most abundant vitamin E form in nature (Brigelius-Flohe & Traber, 1999).

Natural α-tocopherol is a single stereoisomer as the tocopherols synthesised in nature always have the same stereochemical structure, namely the RRR configuration shown in Figure 1 (Jensen & Lauridsen, 2007). However, when α-tocopherol is produced commercially, a racemic mixture of eight possible stereoisomers arises (Weiser & Vecchi, 1981). These stereoisomers are named RRR, RRS, RSS, RSR, SSS, SSR, SRR and SRS after the position of the CH group at the three chiral centres, C2, C4' and C8' (Weiser & Vecchi, 1981). The synthetic form of α-tocopherol is called *all-rac* α-tocopherol and ranges in activity from 21 to 90% relative to RRR α-tocopherol (Weiser & Vecchi, 1982; Brigelius-Flohe & Traber, 1999; DellaPenna, 2005).

General tocopherol structure with RRR configuration

General tocotrienol structure
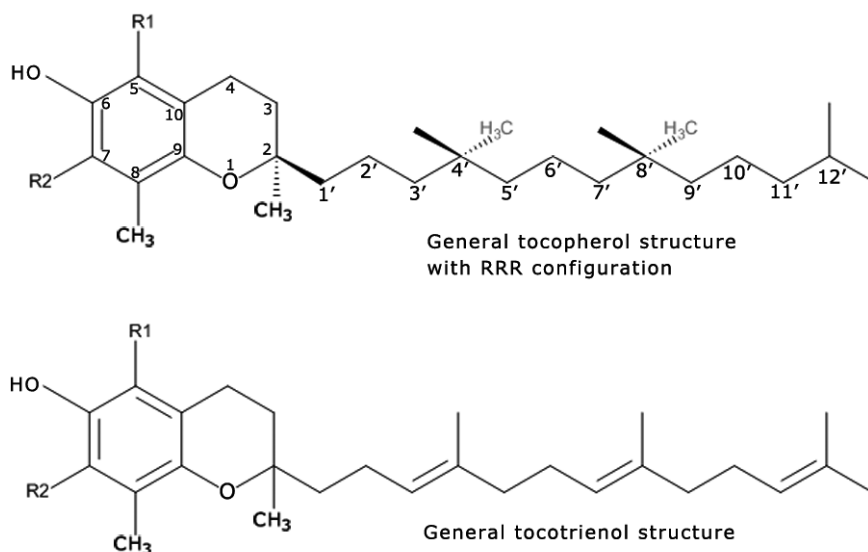
Form	R_1	R_2	*Relative activity (tocol vs. trienol
α-tocopherol/trienol	-CH₃	-CH₃	100% *vs.* 30%
β-tocopherol/trienol	-CH₃	-H	50% *vs.* 5%
γ-tocopherol/trienol	-H	-CH₃	10% *vs.* 0%
δ-tocopherol/trienol	-H	-H	3% *vs.* 0%

Figure 1. General structure of tocopherol and tocotrienol, the tocopherol structure has the RRR configuration. The table indicates the ring methyls in α, β, γ and δ tocopherols and tocotrienols. *Relative activity refers to the vitamin E activity of each tocopherol and tocotrienol with α-tocopherol being 100%. Modified from DellaPenna (2005).

1.1.2 Function of α-tocopherol

α-Tocopherol is a chain-breaking antioxidant and prevents propagation of free radical reactions at an initial step to keep levels of reactive oxygen species (ROS) low (Brigelius-Flohe & Traber, 1999; McDowell, 2000; Holländer-Czytko *et al.*, 2005). This is done by searching for lipid peroxy radicals and quenching or chemically reacting with singlet oxygen and other ROS to make them harmless (DellaPenna & Pogson, 2006). Apart from the chain-breaking antioxidant function, studies on animals suggest that α-tocopherol also influences the regulation of membrane-associated signalling pathways and the modulation of gene expression (Bramley *et al.*, 2000; Falk & Munne-Bosch, 2010).

In plants, the two major roles of α-tocopherol are to protect the chloroplasts against oxidative damage and to protect Photosystem II from photoinhibition,

thus allowing photosynthetic function in thylakoid membranes (Munné-Bosch & Alegre, 2002; Havaux *et al*., 2005).

In humans and animals, α-tocopherol is essential for optimum function of the reproductive, muscular, circulatory, nervous and immune systems (McDowell, 2000). α-Tocopherol protects the cells and cell membranes from ROS, which are continuously produced during normal cellular metabolism. The ROS are necessary for animals as they destroy intracellular pathogens but the products from these reactions and accumulation of ROS can damage healthy cells and have to be regulated by antioxidants. Thus, α-tocopherol is important for the immune system and health (McDowell, 2000; Falk & Munne-Bosch, 2010).

1.1.3 Nomenclature of β-carotene

Carotenoids are present in all photosynthetic organisms. They are isoprenoids and generally consist of eight isoprene units (Hirschberg, 1999; Fraser & Bramley, 2004). This thesis focuses on β-carotene (Figure 2) but over 700 carotenoids are known and have important functions in plants (DellaPenna & Pogson, 2006). Carotenoid species that contain a β-ring can be converted to retinol and are thus precursors for vitamin A (Hirschberg, 1999; DellaPenna & Pogson, 2006).

β-Carotene is the most active precursor for vitamin A as it contains two β-rings and can be cleaved to form two *all-trans*-retinol molecules, which is the alcohol form of vitamin A (Von Lintig, 2010; Rébeillé & Douce, 2011a).
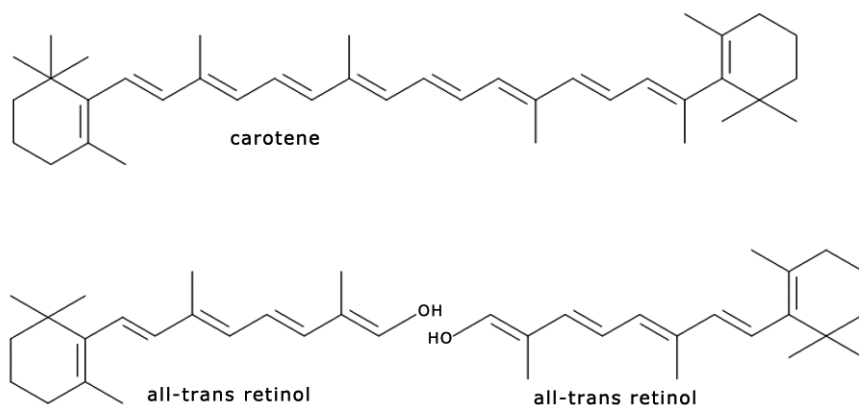

Figure 2. One β-carotene molecule can be cleaved into two *all-trans*-retinol molecules.

1.1.4 Function of β-carotene

β-Carotene like α-tocopherol, is an antioxidant (Asensi-Fabado & Munné-Bosch, 2010). In plants, carotenoids are essential in photosynthesis where they harvest light and protect the plants from free radicals, thus controlling ROS accumulation in plastids together with tocopherols (Hirschberg, 1999; DellaPenna & Pogson, 2006; Triantaphylidès & Havaux, 2009). They are also involved in pigmentation of flowers and fruits (Hirschberg, 1999). When the plants are exposed to excessive light, photoprotective mechanisms are activated including accumulation of antioxidants such as carotenoids and tocopherols. The mechanisms are activated to de-toxify free radicals and repair damaged proteins (Rébeillé & Douce, 2011a).

In human nutrition the ability for conversion from carotenoids to vitamin A is the major importance of carotenoids, but other health benefits are also attributed to the carotenoids (Hirschberg, 1999). In dairy cows, β-carotene and vitamin A are important for reproduction, immune function and health (Michal *et al*., 1994; Kume & Toharmat, 2001). Vitamin A is necessary for supporting growth in animals (Sommer & Davidson, 2002). Deficiency of vitamin A can cause loss of vision and defects in reproduction (McDowell, 2000).

1.2   Synthesis

The synthesis pathways of α-tocopherol and β-carotene are described in detail in several reviews (Munné-Bosch & Alegre, 2002; Fraser & Bramley, 2004; DellaPenna & Pogson, 2006). Figure 3 shows a schematic picture of the pathways in α-tocopherol and β-carotene synthesis. Tocopherols and carotenoids are synthesised in the plastids of the plants and are biosynthetically linked to each other via the five-carbon compounds isopentyl diphosphate (IPP) and geranylgeranyl diphosphate (GGDP), which originate from pyruvate and glyceraldehyde-3-phosphate (Hirschberg, 1999; Fraser & Bramley, 2004; Asensi-Fabado & Munné-Bosch, 2010). In the biosynthesis of carotenoids, GGDP follows the route leading to phytoene, which is used in carotenoid synthesis. In tocopherol synthesis, GGDP is converted to phytyl-diphosphate (PDP), which condensates with homogentisate to eventually form α-tocopherol (Asensi-Fabado & Munné-Bosch, 2010). Tocopherols are amphipathic molecules with the polar head derived from aromatic amino-acid metabolism (homogentisate) and the saturated tail derived from PDP (DellaPenna & Pogson, 2006; Asensi-Fabado & Munné-Bosch, 2010).
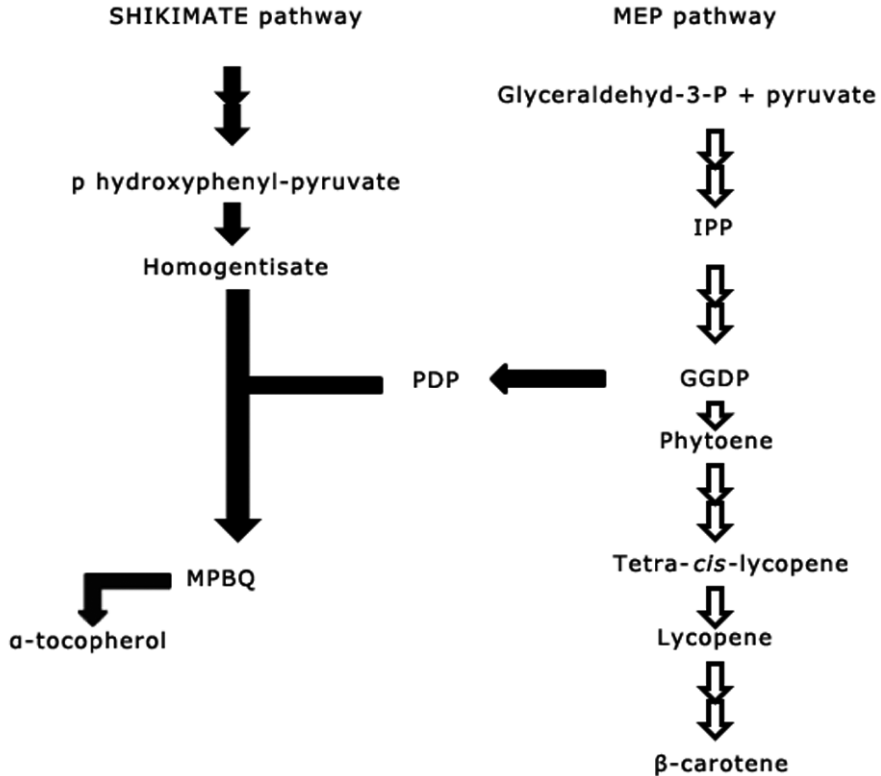
Figure 3. Overview of α-tocopherol and β-carotene biosynthesis in plants. The 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway provides isopentylpyrophosphate (IPP) for synthesis of the central intermediate geranylgeranyl diphosphate (GGDP) of tocopherol and carotenoid synthesis. The white arrows show the pathway of carotenoids and the black arrows the synthesis of tocopherols from homogentisate (a product of the shikimate pathway) and phytyl-diphosphate (PDP). In the synthesis of tocopherol, homogensitate and PDP are condensed to methyl-6-phytyl-1,4-benoquinone (MPBQ) and then converted to α-tocopherol.

1.2.1 α-Tocopherol

α-Tocopherol synthesis in plants is increased by environmental stress, such as high light, wounding, pest attack or senescence (Holländer-Czytko *et al.*, 2005; Munné-Bosch, 2005). During ageing, α-tocopherol production in plants increases to protect them against damage from increased levels of radicals and ROS (Holländer-Czytko *et al.*, 2005; Munné-Bosch, 2005). However, concentrations of α-tocopherol only increase until the stress becomes so severe that the amounts of ROS in the chloroplasts exceed α-tocopherol synthesis, whereupon the α-tocopherol concentrations in the plant decrease (Munné-Bosch, 2005).

1.2.2 β-Carotene

Carotenoid formation is a highly regulated process in plants and is affected by light intensity and stage of development of the plant (Hirschberg, 1999; Fraser & Bramley, 2004). Increases in carotenoid accumulation occur, for example, during fruit ripening and flower development. Increased carotenoid accumulation coincides with increased transcript abundance of some key steps in the synthesis pathway (Rébeillé & Douce, 2011a). Carotenoids and tocopherols have a complementary role in controlling singlet oxygen levels in the chloroplast and act similarly when the plants are exposed to photooxidative stress (Asensi-Fabado & Munné-Bosch, 2010).

1.3 Variations in α-tocopherol and β-carotene concentrations in forages

α-Tocopherol and β-carotene are most abundant in the leaves of forage and the proportion of leaves in plants is therefore considered to have a great effect on α-tocopherol and β-carotene concentrations in forages (Brown, 1953; Ballet *et al*., 2000). The leaf proportion can vary with species, maturity, climate conditions, season and latitude and these factors are known to affect the α-tocopherol and β-carotene content in forages (Olsson *et al*., 1955; Hjarde *et al*., 1963).

Many studies report that α-tocopherol and β-carotene concentrations in forages decrease as plants mature and suggest that this is an effect of the decreasing proportion of leaves in the plant (Brown, 1953; Olsson *et al*., 1955; Ballet *et al*., 2000). However, proportion of leaves is not reported in all published experiments and the timing of the maximum concentration of vitamins during plant maturation is inconsistent between studies.

The α-tocopherol concentration in forages has been shown to increase until the plant starts to flower, followed by a decrease (Hjarde *et al*., 1963). However, Brown (1953) found the highest α-tocopherol concentrations in younger grasses and found decreases from 79 to 90% ranging from the early to the seed maturing stage. Ballet *et al*. (2000) concluded in a review that the highest α-tocopherol and β-carotene concentrations were to be found in the vegetative to ear/bud stages of grasses and legumes and the lowest concentrations after flowering of the plants.

Environmental factors, such as sun-light, photoperiod and temperature, also affect the α-tocopherol and β-carotene contents in plants (Havaux *et al*., 2005). In a study by Olsson *et al*. (1955), red clover, white clover, timothy, meadow fescue and perennial ryegrass was shaded to obtain a fixed photoperiod of 10 hours per day. The shaded swards had higher carotene concentrations than un-

shaded swards. This might be an effect of higher consumption of β-carotene when swards were exposed to light for a longer period and, thus, a lower carotene concentration in plants exposed to excessive light (Havaux *et al.*, 2005; Munné-Bosch, 2005). It has also been shown that high light intensity at low temperatures is more stressful for plants than high light intensity at high temperatures (Havaux *et al.*, 2005).

1.4   Effect of conservation on α-tocopherol and β-carotene concentrations in forages

Ensiling is superior to haymaking in preserving α-tocopherol and β-carotene in forages (Ballet *et al.*, 2000). There can be losses of up to 80-90% of α-tocopherol and β-carotene in hay, but in well-fermented silages the losses are often less than 20% (Carter, 1960).

Vitamin losses increase with poor wilting conditions (Carter, 1960), but less is known about losses during the ensiling process. Losses of α-tocopherol during ensiling vary between studies and both ensiling conditions and choice of plant species affect losses of α-tocopherol and β-carotene during ensiling (Mogensen *et al.* In Press; Beeckman *et al.*, 2010).

Nozière *et al.* (2006) noted that well-fermented silages usually had β-carotene losses of less than 20%, whereas Kalač (1983) reported that there was no clear relationship between silage quality and its β-carotene content. Kalač (1983) also found large differences between plant species in losses of β-carotene during ensiling.

Use of additives can also affect the vitamin content in forage. However, few ensiling studies have evaluated the effect of additives on α-tocopherol and β-carotene concentrations in silage. Kalač and Kyzlink (1979) reported greater losses of β-carotene in red clover ensiled with acidic additives than in untreated silage, whereas Shingfield *et al.* (2005) found that the use of acid or inoculant additives in grass silage resulted in higher contents of α-tocopherol than in untreated grass silage. Nadeau *et al.* (2004) reported inconsistent results on α-tocopherol concentrations of grass-clover silages when additives were used.

1.5   α-Tocopherol and β-carotene in dairy cows

1.5.1 Supply

The large variations in α-tocopherol and β-carotene concentrations in forages have led to the recommendation that the diet of dairy cows should be supplemented with vitamins A and E to fulfil vitamin requirements, regardless of the vitamin concentration of the basal diet (NRC, 2001). The requirements,

expressed in international units (IU), are higher in the dry period than during lactation.

In Sweden, vitamin E and A supplements of 80 and 5500 IU kg^{-1} DM, respectively, are recommended for dry cows. The recommended supplementation of vitamins E and A during lactation is lower, 20 and 3000 IU kg^{-1} DM, respectively (Spörndly, 2003). One IU of vitamin E is defined as the activity of 1 mg *all-rac* α-tocopheryl acetate, while 1 IU of vitamin A is defined as 300 mg retinol or 400 mg β-carotene (McDowell, 2000).

The supplemented vitamins are usually synthetically derived and *all-rac* α-tocopheryl acetate and retinyl acetate are commonly used vitamin supplements of vitamin E and A, respectively. The alcohol forms of the vitamins (retinol and α-tocopherol) are usually esterified to increase the stability of the vitamins (Herdt & Stowe, 1991).

The ambition in organic production is that supplementation of vitamins should be limited and from August 2000 until December 2005 there was a general ban on the use of synthetic supplements in organic ruminant production in the European Union (EU) (EU, 1999). However, due to doubts concerning sufficient natural provision, especially of the fat-soluble vitamins (A, D and E) an exemption from the ban was later implemented in the whole EU.

Supplementation with higher doses of vitamin E has been claimed to improve udder health and studies on this have been conducted worldwide. However, these studies show inconsistent results and it is not clear whether higher supplementation with α-tocopherol improves udder health in dairy cows (Weiss *et al*., 1997; Politis *et al*., 2004; Persson Waller *et al*., 2007; Bouwstra *et al*., 2010).

A number of fertility trials with dairy cows have also been performed in which extra vitamin E has been supplemented to the cows with the aim of improving fertility. Some studies have shown a positive effect on fertility in supplemented cows (Campbell & Miller, 1998; Baldi *et al*., 2000), whereas no effects were found by others (Persson Waller *et al*., 2007; Horn *et al*., 2010).

1.5.2 Natural *vs*. synthetic vitamins

Vitamin E

The semi-natural form, RRR α-tocopheryl acetate, is generally believed to have a higher activity than *all-rac* α-tocopheryl acetate (1.36 *vs*. 1.00 IU mg^{-1}). However, this has been questioned in several studies. Vagni *et al*. (2011) suggest in their review that the activity of *all-rac* α-tocopheryl acetate should be divided by two, as it has been shown that the 2S stereoisomers in synthetic

α-tocopherol are not retained in the blood. The 2S stereoisomers have an S configuration at the chiral centre C2 of α-tocopherol (Weiser *et al*., 1996).

Several studies have shown that the natural form RRR α-tocopherol has higher bioavailability than the synthetic form *all-rac* α-tocopherol (Meglia *et al*., 2006; Weiss *et al*., 2009; Dersjant-Li & Peisker, 2010; Vagni *et al*., 2011). Bioavailability is a measure of the plasma and tissue concentrations of tocopherols and their metabolites (Jensen & Lauridsen, 2007).

As reviewed by Blatt *et al*. (2004), the higher availability of RRR α-tocopherol compared with *all-rac* α-tocopherol is believed to be the result of higher affinity of the 2R forms than of the 2S forms for hepatic α-tocopherol transfer protein (α-TTP). The bioavailability is also thought to be affected by the retention time of α-tocopherol, which is higher for the 2R forms than for the 2S forms, resulting in a higher concentration of the 2R forms than of the 2S forms in most tissues (Blatt *et al*., 2004).

Furthermore, when equal amounts of IU of vitamin E are fed, the concentration of α-tocopherol in biological fluids is usually greater when RRR α-tocopheryl acetate is fed than when *all-rac* α-tocopheryl acetate is fed (Meglia *et al*., 2006; Weiss *et al*., 2009; Dersjant-Li & Peisker, 2010). This further supports the suggestion that the bioavailability, and thus, activity of α-tocopherol should be reconsidered as reviewed by Vagni *et al*. (2011).

Vitamin A

The synthetic form of retinol has been shown to have similar bioavailability to the natural form and therefore synthetic and natural vitamin A activity is defined as being the same (Herdt & Stowe, 1991).

1.5.3 Absorption

α-Tocopherol

Tocopherol and tocotrienol are absorbed in the intestine, where all forms of vitamin E are absorbed without discrimination (Brigelius-Flohe, 2006; Rébeillé & Douce, 2011b). The esterified acetate forms are hydrolysed before absorption and the tocopherols are then packaged into chylomicrons, secreted into the lymph and transported to the liver (McDowell, 2000). In the liver α-tocopherol is specifically selected by the α-TTP for distribution to plasma and peripheral tissues (Brigelius-Flohe, 2006; Rébeillé & Douce, 2011b). Thus, a major reason for the higher bioavailability of α-tocopherol than the other forms of tocopherol and tocotrienol is the higher affinity for α-tocopherol by α-TTP in the liver and other tissues (Brigelius-Flohe, 2006).

β-Carotene

The ability to absorb β-carotene differs between mammals. Rats, pigs, sheep and dogs cleave most β-carotene to retinal in the intestinal mucosa, whereas humans, cattle and horses absorb large amounts of β-carotene (McDowell, 2000). In cattle, there is a strong breed difference in absorption of β-carotene. Cows that are more efficient in converting β-carotene to vitamin A in the intestine have whiter adipose tissue and milk fat than cows that are less efficient (McDowell, 2000).

Dietary vitamin A is often provided as long-chain fatty acid esters of retinol (retinyl esters) and these are hydrolysed to retinol before uptake in the intestine (Harrison, 2012). The free retinol, from either retinyl esters or pro-vitamin A carotenoids, is then re-esterified to retinyl esters, which are incorporated with intact carotenoids into chylomicrons. The chylomicrons are transported to the liver via the lymphatic system. The liver stores vitamin A as retinyl esters, which are hydrolysed to retinol before being transported via the blood stream to other tissues by retinol binding protein (McDowell, 2000; Traber, 2000).

1.5.4 Concentrations of α-tocopherol, β-carotene and retinol in blood

Concentrations of vitamins in blood are often measured in studies to determine adequate and deficient levels in connection with health and fertility traits (Frye *et al.*, 1991; Herdt & Stowe, 1991). The concentration of α-tocopherol, β-carotene and retinol in blood is known to decrease around calving (Goff & Stabel, 1990; Weiss *et al.*, 1990; Vagni *et al.*, 2011) and α-tocopherol concentrations below 2 mg L^{-1} are usually regarded as deficient (Frye *et al.*, 1991). Based on health and fertility studies in cows, Weiss (1999) suggests that plasma levels of α-tocopherol should be above 3-3.5 mg L^{-1} at parturition.

Desirable blood concentrations of β-carotene are considered to be above 3 mg L^{-1} and retinol levels should exceed 3 mg L^{-1}. Retinol levels below 1.5 mg L^{-1} are regarded as deficient (Herdt & Stowe, 1991).

1.5.5 Concentrations of α-tocopherol, β-carotene and retinol in milk

Concentrations of α-tocopherol, β-carotene and retinol in milk and colostrum are dependent on the amounts consumed by the cow (Weiss *et al.*, 1990; Jensen *et al.*, 1999). However, only a small proportion of the vitamins present in the feeds are secreted into the milk (Jensen *et al.*, 1999).

Mogensen *et al.* (In press) found concentrations of 0.82, 0.17 and 0.41 mg α-tocopherol, β-carotene and retinol, respectively, per litre milk in their study in five organic dairy herds in Denmark. The milk was taken from the milk tank, thus represents milk from cows in all stages of lactation. According to Mogensen *et al.* (In press) these concentrations are in agreement with findings

from other studies. Colostrum generally contains higher concentrations of α-tocopherol, β-carotene and retinol (Jensen *et al*., 1999).

Weiss *et al*. (2009) found higher α-tocopherol concentrations in milk from cows fed RRR α-tocopheryl acetate compared with cows fed *all-rac* α-tocopheryl acetate, although the same amount of IU was given. This supports earlier findings that the 2S forms are almost absent in milk (Meglia *et al*., 2006; Jensen & Lauridsen, 2007).

High concentrations of vitamins in the milk are beneficial for milk quality and for calf health (Vagni *et al*., 2011). Results from several studies suggest that vitamin E has a positive effect on both somatic cell count (SCC) and oxidative stability of milk (reviewed by Vagni *et al*., 2011).

2 Aim

The overall aim of the present thesis was to investigate variations in α-tocopherol and β-carotene concentrations in forages as affected by plant and environmental factors and to investigate utilisation of vitamins A and E in organic dairy cows.

Specific objectives were to:

- Study how site, year, cutting date, wilting and ensiling technique affect α-tocopherol and β-carotene concentrations in different forages species.
- Study effects of a large daily dose (2400 IU) of semi-natural vitamin E, supplemented during a limited period around calving on α-tocopherol, β-carotene and retinol concentrations in blood plasma and milk of organic dairy cows during the lactation.

3 Materials and Methods

The following paragraphs provide a short description of the materials and methods used. For a more detailed description, see Papers I-IV.

3.1 Forages

3.1.1 Crops

In Papers I-III, forage mixtures were sown at three different sites in Scandinavia (Figure 4). Two of these sites were in Sweden, Lanna Research Station, SLU, Skara and Röbäcksdalen Research Station, SLU, Umeå (Papers I and III). The third site was at Research Centre Foulum, Tjele, Aarhus University, Denmark (Paper II).
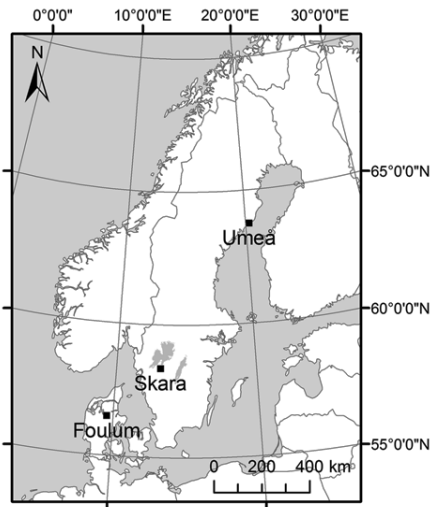

Figure 4. Sites where the forage experiments were performed; Skara and Umeå in Sweden and Foulum in Denmark.

In Sweden, birdsfoot trefoil (*Lotus corniculatus* L), red clover (*Trifoilum pratense* L.), timothy (*Phleum pratense* L.) and meadow fescue (*Festuca pratensis* Huds.) were sown in three mixtures, birdsfoot trefoil + timothy (Bft+Ti), red clover + timothy (Rc+Ti) and red clover + meadow fescue (Rc+Mf). In Denmark, meadow fescue was replaced with perennial ryegrass (*Lolium perenne* L.) and the following mixtures were sown: Bft+Ti, Rc+Ti, red clover + perennial ryegrass (Rc+Pr) and white clover (*Trifolium repens* L.) + perennial ryegrass (Wc+Pr). In the mixture with birdsfoot trefoil in Denmark, a large proportion of unsown white clover was present in the sward and thus the Bft+Ti forage is referred to hereafter as Bft+Wc+Ti.

Forage mixtures in the animal study presented in Paper IV consisted of legume-grass mixtures of timothy, meadow fescue, white clover and red clover.

3.1.2 Establishment

The forage mixtures in Papers I, II and III were established as undersown crops in barley in spring 2004, and at Skara the forage mixtures were re-established in spring 2006. This resulted in forage mixtures from two first-year leys at Skara (2005 and 2007) and forage mixtures from first-year and second-year leys at Umeå and in Denmark (2005 and 2006, respectively).

Fertilisation of the soils and soil types are described in Papers I, II and III.

3.1.3 Climate

Data on precipitation, temperature and solar radiation at Skara and Umeå during the study period were obtained from the Swedish Meteorological and Hydrological Institute throughout the experimental period (Figure 1a-d in Paper I). Precipitation and temperature were recorded daily at the Research Centre Foulum, approximately 200 m from the experimental area. Precipitation and daily mean temperature at the site in Denmark during the study are presented in Paper II.

3.1.4 Harvest

The three forage mixtures used in Paper I were cut on three different occasions during spring growth; at one week before expected heading (early), at heading (mid) and at one week after heading of timothy (late).

Forage for the ensiling study in Paper III was harvested as regrowth eight weeks after the mid cut at Skara in 2005 (11 August). The plots in Denmark (Paper II) were harvested in a four-cut system in both years.

Forage mixtures used in the animal study in Paper IV were harvested in a two-cut system in both years.

3.1.5 Sampling

Samples from all plots in Sweden were cut by hand at a stubble height of 3-7 cm on the three cutting dates in spring growth. One sample from each plot and cutting date was sorted into sown legumes, unsown legumes, sown grasses, weeds and dead plant material. Another sample was sorted to determine proportion of leaf blades for all forage species. All samples were dried in a forced air drying oven to determine dry weights. A 0.5 m long row was cut by hand and sorted into phenology classes according to a scale devised by (Zadoks *et al.*, 1974) and Gustavsson (2011).

Samples for analysis of α-tocopherol and β-carotene concentrations in all species and at all cutting dates were frozen at -20 °C immediately after sorting, within 3 (Umeå) to 48 hours (Skara) of cutting. Samples for analysis of other nutrients were also frozen at -20 °C after species separation. All samples were kept at 4 °C until separation and drying/freezing.

In Denmark (Paper II), samples for analysis of α-tocopherol and β-carotene and determination of botanical composition were taken from species in all mixtures at all cuts, whereas samples for analysis of other nutrients were taken from two replicates in harvests 1, 3 and 4.

Forages from the regrowth for ensiling in Paper III were harvested at a stubble height of 8 cm. Representative samples of fresh matter were collected from each mixture, chopped in a machine to 12 mm length and dried outdoors for 3 hours on plastic sheeting. Samples for analysis of vitamins were taken from unwilted herbage, wilted herbage and all silage treatments. Samples for analysis of other nutrients were taken from wilted herbage and silages.

Sampling of forages for the animal study in Paper IV is described in section 3.2.3.

3.1.6 Ensiling

After wilting, the three forage mixtures in Paper III were exposed to one of three treatments in which an acid and an inoculant additive were compared with a control treatment without any additive. The forage mixtures from the three treatments were ensiled in 1.7 L mini silos, equipped with fermentation traps, and stored indoors (20 ºC) for 100 days before being opened.

Forage mixtures in the animal study in Paper IV were wilted and ensiled in bunker silos without additives.

3.2   Dairy cows and housing

The herd used for Paper IV consisted of around 70 Swedish Holstein dairy cows. The study was divided into experiment (Exp) 1 (October 2005 to

November 2006), which included 44 cows, and Exp 2 (December 2006 to November 2007), which included 32 cows. The cows were milked twice a day and were housed in a loose housing system with cubicles and concrete floor. The cows were out on pasture from 09.00 to 16.00 from mid-May to the end of October.

3.2.1 Vitamin treatment

The vitamin treatments used were basal natural vitamins from a 100% organic diet supplemented with 0 (C) or 2400 IU RRR-α-tocopheryl acetate during the transition period around calving (E).

The supplementation with 2400 IU RRR-α-tocopheryl acetate started three weeks before expected calving (BEC) and lasted until three weeks post-calving (PC). In Exp 1, all cows consumed a diet without synthetic vitamins, but in Exp 2 the synthetic vitamins A, D and E were supplemented to all cows throughout the experiment according to Swedish recommendations for lactating cows.

3.2.2 Diets

The cows were fed a partly mixed ration. The diet consisted of grass-clover silage, barley, cold-pressed rapeseed cake, field beans or peas and minerals. Pasture intake was estimated based on the hours the cows were allowed to graze. Feed rations and nutritional compositions of the diets are presented in Tables 1 and 2 in Paper IV.

3.2.3 Feed sampling

Silage, pasture and concentrate samples were taken continuously, immediately frozen and analysed for α-tocopherol, β-carotene, crude protein (CP), neutral detergent fibre (NDF), starch and crude fat content (Table 4 in Paper IV). Fermentation characteristics were determined in silage.

3.2.4 Blood and milk sampling and milk recording

Individual blood samples from the tail vein were collected at three weeks BEC, within 24 hours PC, at three weeks PC and between five to seven months PC. After centrifugation, blood plasma was stored at -20ºC until vitamin analysis.

Individual milk samples were taken from the milk recorder jar at the first milking PC within 15 hours PC (colostrum), at four days PC, at three weeks PC and between five to seven months PC. Samples were stored at -20ºC until analysis of vitamins.

Milk yield was recorded individually every second week during the first three months of lactation, and thereafter once a month up to 305 days of

lactation. Simultaneously, individual milk samples were taken for analysis of milk composition (fat, protein, urea and SCC).

3.2.5 Body weight, body condition score, fertility and health

Body weight (BW) of the cows was recorded once a month, and body condition score (BCS) was recorded simultaneously by one trained scorer, using a visual appraisal method (Edmonson *et al.*, 1989).

Fertility of the cows was determined as the number of artificial inseminations (AI) per pregnancy, number of days between calving and the first AI, number of days between calving and the last AI, and calving interval in Exp 2. In Exp 1, only calving interval was recorded, as a bull was used instead of AI for most cows.

Health of the cows was determined by the number of veterinary treatments and by SCC in milk during the first three months of lactation and from three months of lactation up to 305 days of lactation.

3.3 Analyses

3.3.1 Analyses of α-tocopherol and β-carotene

Analyses of α-tocopherol and β-carotene were performed at Aarhus University, Department of Animal Sciences, Research Centre Foulum, Denmark. Forages and concentrates were analysed according to Jensen *et al.* (1998) and concentrations of retinol, β-carotene and α-tocopherol in plasma and milk were determined as previously described (Jensen, 1994; Jensen & Nielsen, 1996).

3.3.2 Analyses of milk composition and other nutrients in forages

Milk composition in Paper IV was analysed by Eurofins Steins Laboratory Inc, Jönköping, Sweden.

Forage samples for Papers I and II were dried or freeze-dried and analysed according to the Cornell Net Carbohydrate and Protein System (CNCPS-system; Sniffen *et al.*, 1992). The analyses were conducted in a commercial laboratory in the USA that specialises in CNCPS analysis (Dairy One, Ithaca, NY, USA) and references to the analyses can be found in Papers I and II.

Nutritional analyses for Papers III and IV were conducted at Eurofins Laboratory, Lidköping, Sweden, as described by Nadeau *et al.* (2007). Weight loss of initial DM of the forage during storage in Paper III was calculated according to Weissbach (2005).

3.4 Statistics

Data from Papers I and III were analysed as a split-split-plot and split-plot design using the mixed model procedure (Paper I) and general linear model procedure (Paper III) of SAS (2001). In the analysis, trial (site/year) was treated as the main plot, species as the subplot and cutting date as the sub-sub-plot in the split-split-plot design of Paper I. In the split-plot analysis in Paper I/III, forage species/mixture was treated as the main plot, and cutting date/forage treatment as the subplot.

Vitamin concentrations of the forage in Paper II and vitamin concentrations in blood and milk, milk yield, milk composition, BW and BCS in Paper IV were analysed as a randomised complete block using proc mixed in SAS (2001). In the analysis of milk yield, milk composition, BW, BCS and SCC, parity (primiparous or multiparous) was included as a covariate.

Fertility variables were analysed using Wilcoxon Two-Sample Test in SAS (2001) and health was analysed using the Fisher exact test.

4 Results

4.1 Forage studies, Papers I-III

The most important results are summarised in these sections, while full details can be found in the individual papers. Paper I includes tables of vitamin concentrations and proportions of leaf blade in forage species harvested during spring growth at Umeå and Skara (Tables 3-6). Paper II includes tables of vitamin concentrations in forages harvested in Denmark (Tables 3-6).

4.1.1 Yield and developmental stages

Averaged over mixtures and cuts in spring growth (Figure 5a), yield was larger for Skara 2005 than for Umeå 2005 and Skara 2007 (P = 0.002). Mixtures with red clover had larger yields than the mixture with birdsfoot trefoil when averaged over trials and cuts (P < 0.0001). For Umeå 2006, the Rc+Ti mixture yielded more than the Rc+Mf and Bft+Ti mixtures, which did not differ (P < 0.0001). The DM yield increased with later cutting date in spring growth averaged across trials and mixtures for Umeå 2005 and Skara 2005 and 2007. The same was observed for Umeå 2006 (P < 0.0001).

In Denmark (Figure 5b), the mixture with birdsfoot trefoil had the lowest annual DM yield in 2005 (P < 0.0001). In 2006, only the red clover mixtures had higher annual DM yield than the Bft+Wc+Ti mixture (P < 0.0001).
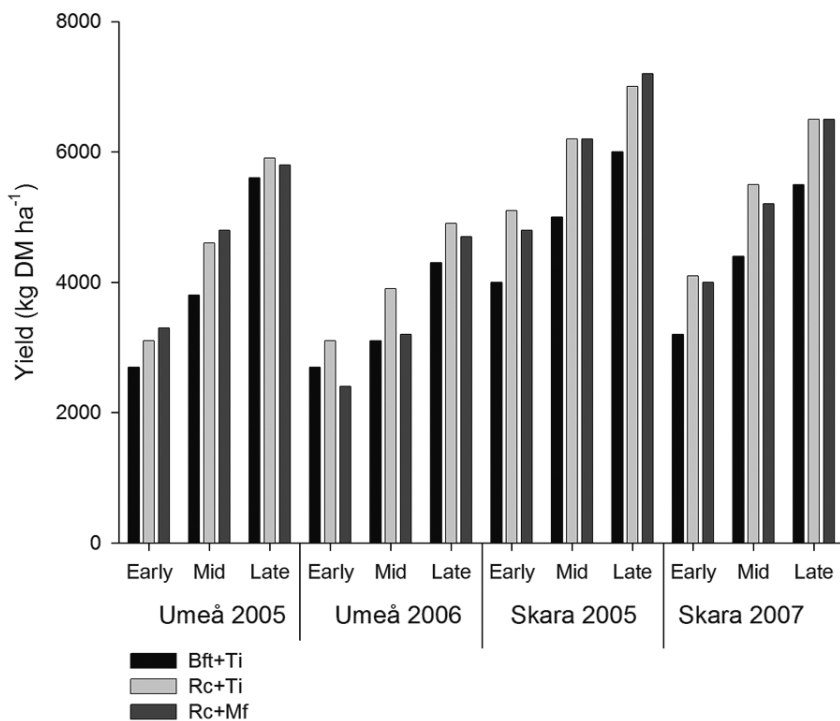
Figure 5a. Yield (kg DM ha^{-1}) of the forage mixtures birdsfoot trefoil+timothy (Bft+Ti), red clover+timothy (Rc+Ti) and red clover+meadow fescue (Rc+Mf) harvested at Skara (2005 and 2007) and at Umeå (2005 and 2006) in Sweden on three occasions in the spring growth (early, mid, late).
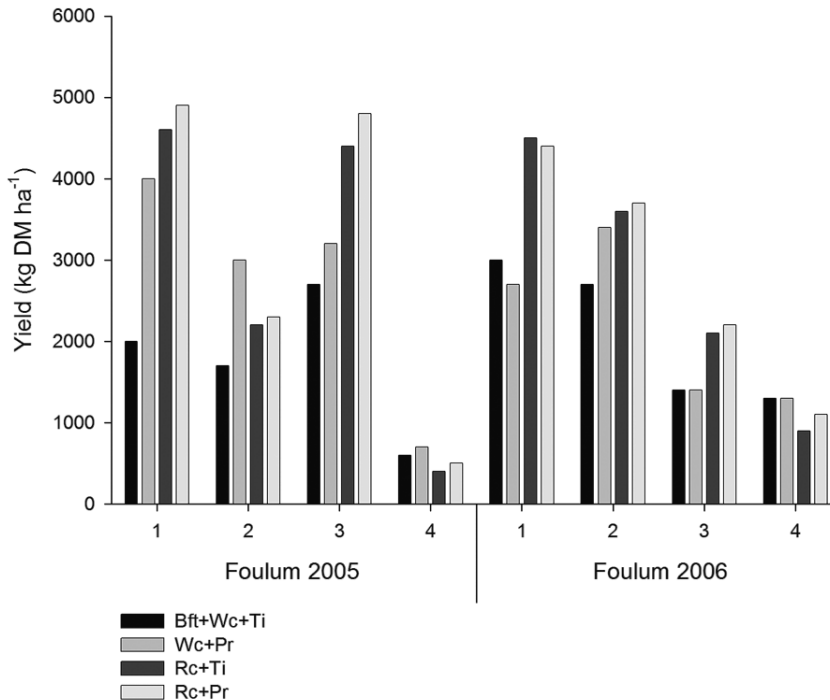
Figure 5b. Yield (kg DM ha^{-1}) of the forage mixtures birdsfoot trefoil+white clover+timothy (Bft+Wc+Ti), white clover+perennial ryegrass (Wc+Pr), red clover+timothy (Rc+Ti) and red clover+perennial ryegrass (Rc+Pr) harvested in a four-cut system at Foulum, in Denmark in 2005 and 2006.

As shown in Figures 6a-d, developmental stages varied between species, sites and years. Variations in developmental stages of birdsfoot trefoil were larger at Skara than Umeå, whereas, red clover seemed to have larger differences in maturity stages at Umeå than Skara. Most of the red clover was in the extended internode stage at Skara, whereas red clover at Umeå ranged from leaf stage to early bloom. There seemed to be only small differences in developmental stages of legumes between years at Skara. Grasses at Umeå in 2005 seemed to have reached a later developmental stage than grasses at Skara in 2005 with a larger proportion of grasses having reached full heading stage at Umeå. Variations in developmental stages between grass species were more inconsistent than between legumes.
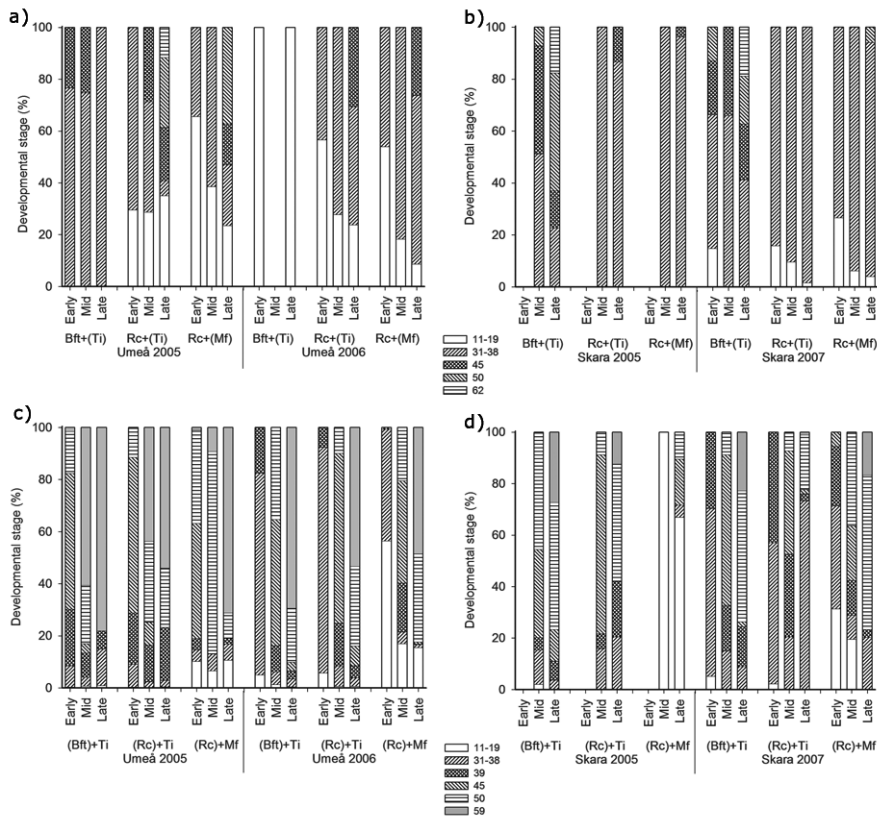
Figure 6. Developmental stages of the **legumes** birdsfoot trefoil (Bft) and red clover (Rc) grown in mixtures with timothy (Bft+Ti and Rc+Ti) and meadow fescue (Rc+Mf) at (a) Umeå in 2005 and 2006 and at (b) Skara in 2005 and 2007. Stages 11-19 represent leaf stage, 31-38 extended internodes, 45 buds enclosed by leaves, 50 visible buds and 62 early bloom. Developmental stages of the **grasses** timothy (Ti) and meadow fescue (Mf) at (c) Umeå in 2005 and 2006 and at (d) Skara 2005 and 2007. Stages 1-19 represent leaf development, 31-38 stem elongation, 39 flag leaf, 45 the tip of the inflorescence in the flag leaf sheath (booting), 50 beginning of heading, and 59 full heading. Cuts were taken on three occasions in spring growth (early, mid and late).

4.1.2 α-Tocopherol concentration

Legumes

The α-tocopherol concentrations in birdsfoot trefoil for Skara 2005 ranged from 35.9 to 65.6 mg kg^{-1} DM. They did not differ from concentrations observed for Skara 2007 and were generally higher than those in birdsfoot trefoil grown at Umeå in 2005, which ranged from 26.9 to 35.5 mg kg^{-1} DM. The α-tocopherol concentration in red clover was similar between trials at

Umeå 2005, Skara 2005 and 2007, and ranged from 15.2 to 31.5 mg kg^{-1} DM ($P = 0.006$).

The α-tocopherol concentration in birdsfoot trefoil was generally higher than that in red clover for all cuts within trials ($P = 0.006$). The concentrations in birdsfoot trefoil and red clover were 31.7 and 17.2 mg kg^{-1} DM respectively for Umeå 2005, 50.4 and 24.6 mg kg^{-1} DM for Skara 2005 and 51.4 and 25.3 mg kg^{-1} DM for Skara 2007, averaged over cuts. For Umeå 2006, birdsfoot trefoil only had higher α-tocopherol concentration than red clovers in the late cut (49.3 *vs*. 15.4 mg kg^{-1} DM; $P = 0.014$).

At Skara, the α-tocopherol concentration in birdsfoot trefoil decreased with later cutting date (from 65.6 to 35.9 mg kg^{-1} DM in 2005 and from 56.0 to 44.7 mg kg^{-1} DM in 2007), whereas the α-tocopherol concentrations in red clover were generally similar between cuts in all trials ($P = 0.006$). For Umeå 2006, the α-tocopherol concentration of red clover varied more between cuts and ranged from 14.1 to 53.3 mg kg^{-1} DM in Rc+Ti and from 16.7 to 58.8 mg kg^{-1} DM in Rc+Mf ($P = 0.014$). The α-tocopherol concentration of birdsfoot trefoil did not decrease with later cutting date in any year at Umeå.

In Denmark too, birdsfoot trefoil had high α-tocopherol concentration. In 2005, birdsfoot trefoil had a higher α-tocopherol concentration than the clovers in harvest 2 (74.1 *vs*. 34.8 mg kg^{-1} DM; $P = 0.003$) and harvest 3 (70.6 *vs*. 20.2 mg kg^{-1} DM; $P < 0.0001$). The trend was similar in harvest 4, where birdsfoot trefoil tended to have a higher α-tocopherol concentration than the clovers (118.4 *vs*. 63.9 mg kg^{-1} DM; $P = 0.053$).

In 2006, birdsfoot trefoil had a higher α-tocopherol concentration than the other legumes in all harvests (64.1 *vs*. 24.1, 66.8 *vs*. 25.3, 91.3 *vs*. 26.6 and 138.5 *vs*. 37.6 mg kg^{-1} DM in harvests 1, 2, 3 and 4, respectively; $P < 0.0001$). Red clover had a lower α-tocopherol concentration than white clover in harvest 1 (18.4 *vs*. 35.5 mg kg^{-1} DM) and harvest 2 (21.3 *vs*. 33.2 mg kg^{-1} DM), but there were no differences in α-tocopherol concentration between the clovers in harvests 3 and 4 ($P < 0.0001$).

Grasses

In 2005, all grass species grown at Skara had higher α-tocopherol concentrations than grasses grown at Umeå, when averaged over cuts ($P < 0.0001$), with the concentration ranging from 39.0 to 73.5 mg kg^{-1} DM at Skara and from 27.6 to 35.8 mg kg^{-1} DM, at Umeå. Meadow fescue differed in α-tocopherol concentration between Skara 2005 and 2007 as a mean over cuts (73.5 *vs*. 48.6 mg kg^{-1} DM). Averaged over cuts, timothy grown with red clover had a lower α-tocopherol concentration than meadow fescue (27.6 *vs*.

35.8, 43.1 *vs.* 73.5 and 39.0 *vs.* 48.6 mg kg^{-1} DM for Umeå 2005, Skara 2005 and Skara 2007, respectively).

At Umeå in 2006, timothy had higher α-tocopherol concentration than meadow fescue in the early cut (51.8 *vs.* 20.7 mg kg^{-1} DM). In the mid cut, timothy grown with birdsfoot trefoil had higher α-tocopherol concentration than the other grasses (71.6 *vs.* 16.9 mg kg^{-1} DM), whereas no differences were found between grasses in the late cut ($P = 0.004$).

α-Tocopherol concentration as a mean for the grasses decreased with later cutting date in Skara with most pronounced differences in 2007 (from 59.8 to 32.0 mg kg^{-1} DM in early to late cut; $P < 0.001$).

At Umeå in 2006, α-tocopherol concentration of timothy grown with birdsfoot trefoil decreased in the late cut (from 71.6 to 38.6 mg kg^{-1} DM), whereas the concentration in meadow fescue increased in the late cut compared with the earlier cuts (from 15.5 to 58.3 mg kg^{-1} DM). Timothy grown with red clover had lower α-tocopherol concentration in the mid cut than in the early and late cuts (18.3 *vs.* 40.0 mg kg^{-1} DM; $P = 0.004$).

In Denmark in 2005, the α-tocopherol concentration was lower in perennial ryegrass grown with red clover than in the other grasses in the first harvest (42.2 *vs.* 58.4 mg kg^{-1} DM; $P = 0.038$). In 2006, timothy from the Bft+Wc+Ti mixture had a higher α-tocopherol concentration than perennial ryegrass grown with red clover in the first harvest (47.5 *vs.* 26.7 mg kg^{-1} DM; $P = 0.016$).

Timothy from the mixture Bft+Wc+Ti had a higher α-tocopherol concentration than the other grasses in the second harvest (63.0 *vs.* 34.4 mg kg^{-1} DM; $P = 0.004$) in Denmark in 2005. In 2006, timothy from Bft+Wc+Ti had a higher α-tocopherol concentration than perennial ryegrass and timothy from the red clover mixtures (71.4 *vs.* 47.0 mg kg^{-1} DM; $P < 0.001$). In the fourth harvest in Denmark in 2005, timothy from the Bft+Wc+Ti mixture had a higher α-tocopherol concentration than perennial ryegrass grown with red clover (142.4 *vs.* 80.8 mg kg^{-1} DM; $P = 0.035$) and timothy from both mixtures had a higher α-tocopherol concentration than perennial ryegrass in 2006 (112.9 *vs.* 65.0 mg kg^{-1} DM; $P < 0.001$).

Mixtures and silage

Regrowth mixtures in the silage study did not differ in α-tocopherol concentration in either the unwilted or wilted herbage. Wilting decreased α-tocopherol concentration in the Bft+Ti mixture but not in the red clover mixtures (Figure 7a).

During ensiling, the α-tocopherol concentration decreased in the acid-treated red clover mixtures, whereas the α-tocopherol concentration of the Bft+Ti mixture increased during ensiling for both the control and additive-

treated silages. However, there were no differences in α-tocopherol concentration between silage treatments in the Bft+Ti mixture, whereas inoculated silages of red clover mixtures had higher α-tocopherol contents than both the control and acid-treated red clover silages (Figure 7a). All silages were well fermented (Table 4 in Paper III).
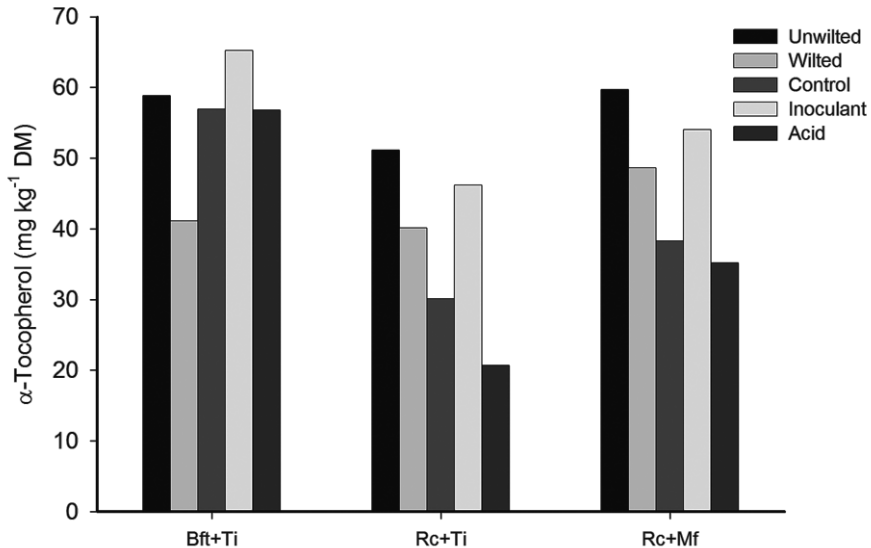

Figure 7a. α-Tocopherol concentration in unwilted and wilted forages, untreated (control) and inoculant or acid treated silages of the mixtures birdsfoot trefoil+timothy (Bft+Ti), red clover+timothy (Rc+Ti) and red clover+meadow fescue. The forage was harvested as a regrowth at Skara, Sweden 11 August in 2005.

4.1.3 β-Carotene concentration

Legumes

Birdsfoot trefoil had a higher β-carotene concentration for Skara 2005 compared with Umeå 2005 when averaged over cuts in the spring growth (69.6 *vs*. 54.9 mg kg^{-1} DM; P = 0.037). β-Carotene concentration of red clover grown at Skara and Umeå in 2005 did not differ when averaged over cuts and ranged from 38.6 to 48.5 mg kg^{-1} DM. Birdsfoot trefoil and red clover grown with meadow fescue had higher β-carotene concentration for Skara 2007 than Skara 2005, when averaged across cuts (82.1 *vs*. 69.6 in birdsfoot trefoil and 54.8 *vs*. 42.9 mg kg^{-1} DM in red clover; P = 0.037).

Averaged over species, the mid cut for Skara 2005 had higher β-carotene concentrations than the early and late cuts, which did not differ (68.3 *vs*. 46.4 mg kg^{-1} DM). At Skara in 2007 and Umeå in 2006, the β-carotene

concentration decreased with later cutting date from 69.4 to 56.2 mg kg^{-1} DM for Skara 2007 ($P < 0.001$) and from 44.5 to 31.5 mg kg^{-1} DM for Umeå 2006 ($P = 0.027$).

In Denmark in 2005, β-carotene concentration was higher in birdsfoot trefoil than in the clovers in harvest 3 (60.1 *vs.* 32.2 mg kg^{-1} DM; $P < 0.0001$) and harvest 4 (196.4 *vs.* 123.9 mg kg^{-1} DM; $P < 0.001$). In harvest 3, the red clovers had lower β-carotene concentration than white clover (25.2 *vs.* 46.1 mg kg^{-1} DM).

For Denmark 2006, birdsfoot trefoil and white clover had similar β-carotene concentrations in harvests 1, 3 and 4 ranging from 46.5 to 78.3 mg kg^{-1} DM. The concentrations were higher than in red clover in the first harvest (67.0 *vs.* 26.7 mg kg^{-1} DM; $P = 0.002$), whereas no differences in β-carotene concentration were found between the clovers in the third harvest ($P = 0.028$). In the fourth harvest, birdsfoot trefoil and white clover had higher β-carotene concentrations than red clover grown with timothy (73.6 *vs.* 33.1 mg kg^{-1} DM; $P = 0.004$).

Grasses

All grasses grown at Skara in 2005 had higher β-carotene concentration than those grown at Umeå in 2005 and Skara in 2007, when averaged over cuts, with concentrations ranging from 22.0 to 23.4 mg kg^{-1} DM for Umeå 2005, from 42.9 to 52.8 mg kg^{-1} DM for Skara 2005 and from 23.2 to 36.2 mg kg^{-1} DM for Skara 2007 ($P = 0.001$).

Meadow fescue had a higher β-carotene concentration than timothy for Skara 2005 (52.8 *vs.* 43.0 mg kg^{-1} DM), whereas no differences were found between species for Umeå 2005. Timothy grown with birdsfoot trefoil had a lower β-carotene concentration than the other grasses grown at Skara in 2007 ($P = 0.001$).

When averaged over species, β-carotene concentrations decreased with later cuts at Skara (from 46.6 in the early cut to 38.6 mg kg^{-1} DM in the late and from 35.3 in early to 23.9 in late for Skara 2005 and 2007, respectively; $P < 0.004$).

For Denmark 2005, β-carotene concentrations were lower in timothy from the Bft+Wc+Ti mixture than in the other grasses in the first harvest (19.8 *vs.* 34.0 mg kg^{-1} DM; $P = 0.012$). In the third harvest, perennial ryegrass grown with white clover had a higher β-carotene concentration than timothy (58.6 *vs.* 30.8 mg kg^{-1} DM; $P = 0.014$). In the fourth harvest, timothy from the Bft+Wc+Ti mixture had a higher β-carotene concentration than timothy grown with red clover (180.4 *vs.* 120.0 mg kg^{-1} DM; $P = 0.048$).

The only significant difference in β-carotene concentration between the grasses grown in Denmark in 2006 was found in the third harvest, where perennial ryegrass grown with white clover had a higher β-carotene concentration than timothy grown in the Bft+Wc+Ti mixture (67.0 *vs*. 43.2 mg kg^{-1} DM; *P* = 0.018).

Mixtures and silage

No significant interactions in β-carotene concentration were found between treatments and mixture. Averaged over treatments red clover mixtures had lower β-carotene concentrations than the Bft+Ti mixture. Averaged over mixtures, acid-treated silage had lower β-carotene content than unwilted herbage (Figure 7b).
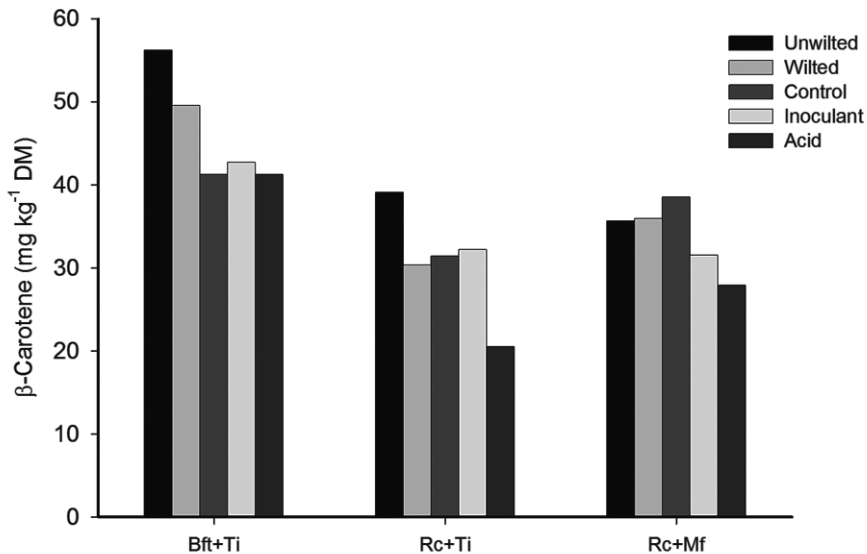
Figure 7b. β-Carotene concentration in unwilted and wilted forages, untreated (control) and inoculant or acid treated silages of the mixtures birdsfoot trefoil+timothy (Bft+Ti), red clover+timothy (Rc+Ti) and red clover+meadow fescue. The forage was harvested as a regrowth at Skara, Sweden 11 August in 2005.

4.1.4 Leaf blade proportion

Legumes

Averaged over trials, birdsfoot trefoil had a higher proportion of leaf blades than red clover in the early and mid cuts (0.53 *vs*. 0.42 and 0.45 *vs*. 0.37, respectively; *P* = 0.004). No significant difference in leaf blade proportion between birdsfoot trefoil (0.37) and red clover grown with meadow fescue

(0.35) were found in the late cut, averaged over trials. Leaf blade proportion was highest in the early cut and decreased with cutting date for both birdsfoot trefoil and red clover, averaged across trials.

Averaged over cuts, birdsfoot trefoil had a higher proportion of leaf blades than red clovers in Umeå 2006 (0.58 *vs*. 0.47; P = 0.006). Averaged over species leaf blade proportion for Umeå 2006 decreased with later cutting date (0.60 *vs*. 0.49 *vs*. 0.41 in early, mid and late cut; $P < 0.0001$).

Grasses

The proportion of leaf blades for timothy ranged from 0.26 to 0.38 in the three trials averaged over cuts, but differences were only found between timothy grown at Umeå in 2005 and Skara in 2007 ($P < 0.001$). Meadow fescue had higher leaf blade proportion at Skara in 2005 (0.60) than at Umeå 2005 (0.29) and at Skara in 2007 (0.41; $P < 0.001$).

Leaf blade proportion between grasses within trials was similar for Umeå 2005 and for Skara 2007 and ranged from 0.26 to 0.29 at Umeå and 0.37 to 0.41 at Skara ($P < 0.001$). At Skara in 2005, meadow fescue had a higher leaf blade proportion than timothy (0.60 *vs*. 0.30; $P < 0.001$). Meadow fescue had a higher leaf blade proportion than timothy at Umeå in 2006, when averaged over cuts (0.46 *vs*. 0.37; $P < 0.0001$).

Leaf blade proportion decreased with later cutting date in all grass species averaged over trials from 0.38 to 0.22, from 0.41 to 0.24 and from 0.57 to 0.31 in Bft+Ti, Rc+Ti and Rc+Mf, respectively ($P = 0.042$).

At Umeå in 2006, leaf blade proportion also decreased with later cutting date, when averaged over species (0.57, 0.38 and 0.24 in early, mid and late cut, respectively; $P < 0.0001$).

4.2   Dairy cow study, Paper IV

4.2.1 Vitamins from the diet

The silage used in Exp 1 contained nearly three times as much α-tocopherol and β-carotene as the silage used in Exp 2, which led to differences in the basal intake of α-tocopherol and β-carotene in the total diets (Table 3 and 4 in Paper IV). The diet in Exp 1 contained twice the concentration of natural α-tocopherol and β-carotene than the diet in Exp 2. However, the supplementation of synthetic α-tocopherol increased α-tocopherol concentration in the diets in Exp 2 to a similar concentration as to that in Exp 1.

4.2.2 Vitamins in blood plasma

The E cows tended to have higher α-tocopherol concentrations in blood plasma at calving and had significantly higher α-tocopherol concentrations in plasma at three weeks PC than the C cows in both experiments (Figure 8a-c). The C cows in Exp 1 had significantly higher concentrations of β-carotene and a tendency for higher retinol concentration in the plasma at calving than the E cows. In Exp 2, C cows tended to have a higher retinol concentration in plasma than the E cows at three weeks PC.

4.2.3 Vitamins in milk

There were no statistical differences in vitamin concentrations of the milk between the treatments in Exp 1 (Figure 8d-f). In Exp 2, the E cows had significantly higher concentrations of α-tocopherol in the colostrum, in the milk at four days PC and at three weeks PC than the C cows. Furthermore, the E cows had significantly higher concentrations of β-carotene in the colostrum and in the milk at four days PC and a tendency for higher β-carotene concentrations in the milk at three weeks PC than the C cows. The E cows also had a higher concentration of retinol in the milk four days PC than the C cows.
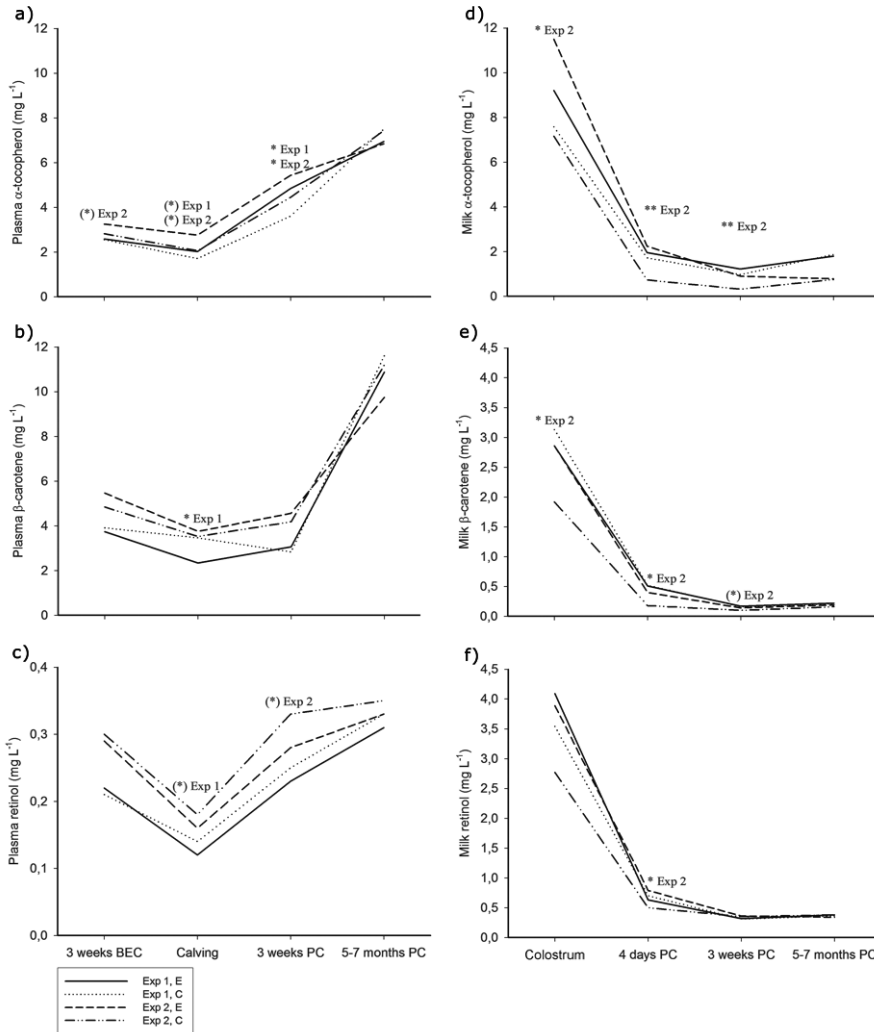
Figure 8. Concentrations of (a, d) α-tocopherol, (b, e) β-carotene and (c, f) retinol of blood plasma (at three weeks before expected calving (BEC), at calving, three weeks post calving (PC) and five to seven months PC) and milk (colostrum, four days PC, three weeks PC and five to seven months PC) in dairy cows. One treatment group received a high dose (2400 IU) of semi-natural vitamin E during the transition time around calving (E) and the other group did not receive this supplement (C). Experiment (Exp) 1 was performed in 2005-2006 and Exp 2 in 2006-2007.

4.2.4 Milk production, milk composition and body condition

Effects of vitamin treatment on milk production, milk composition and body condition are presented in Table 7 in Paper IV.

4.2.5 Fertility and health

No effects of vitamin treatment were found on fertility or health.

5   General Discussion

5.1   Forages, Papers I-III

5.1.1 Site

Differences in α-tocopherol and β-carotene concentrations in birdsfoot trefoil, timothy and meadow fescue grown at Skara in 2005 than Umeå in 2005 were in agreement with earlier studies, where higher vitamin concentrations were found in forages grown in southern of Sweden compared with northern (Olsson *et al*., 1955; Hjarde *et al*., 1963). The effect of site can potentially be caused by interactions of temperature, solar radiation, photoperiod, growth rate, season and leaf blade proportion, which are all known to affect α-tocopherol and β-carotene concentrations in plants (Brown, 1953; Booth & Hobson-Frohock, 1961; Ballet *et al*., 2000; Havaux *et al*., 2005). The lack of differences in vitamin concentrations of red clover between the sites could be a cultivar effect. Different varieties of red clover were used at the two sites as the variety was chosen based on adaptation to the site.

Photoperiod and solar radiation

Photoperiod is believed to affect α-tocopherol and β-carotene concentrations in plants, as swards shaded to obtain 10 hour photoperiod per day have been shown to have a higher vitamin concentration than un-shaded swards (Olsson *et al*., 1955; Hjarde *et al*., 1963).

The similar daily solar radiation at Umeå and Skara could be a result of higher light intensities at Skara than Umeå. However, when light intensities were compared during the cuts, they were similar between the sites in 2005. Higher light intensities have been shown to decrease α-tocopherol and β-carotene concentrations in plants when the consumption of antioxidants exceeds production (Munné-Bosch, 2005).

Havaux *et al.* (2005) found that α-tocopherol concentrations were often higher in plants exposed to high light intensity at high temperature than in plants exposed to high light intensity at low temperatures. High light intensities at low temperatures are believed to be more stressful for plants (Havaux *et al.*, 2005). However, there were not higher light intensity and lower temperature during the cuts at Umeå than at Skara in 2005 indicating that this was not a reason for the lower vitamin concentrations at Umeå.

The longer photoperiod at Umeå than Skara during the growing season (Figure 9) could have affected the growth rate and development of the plants, which are known to affect α-tocopherol and β-carotene concentration in plants.
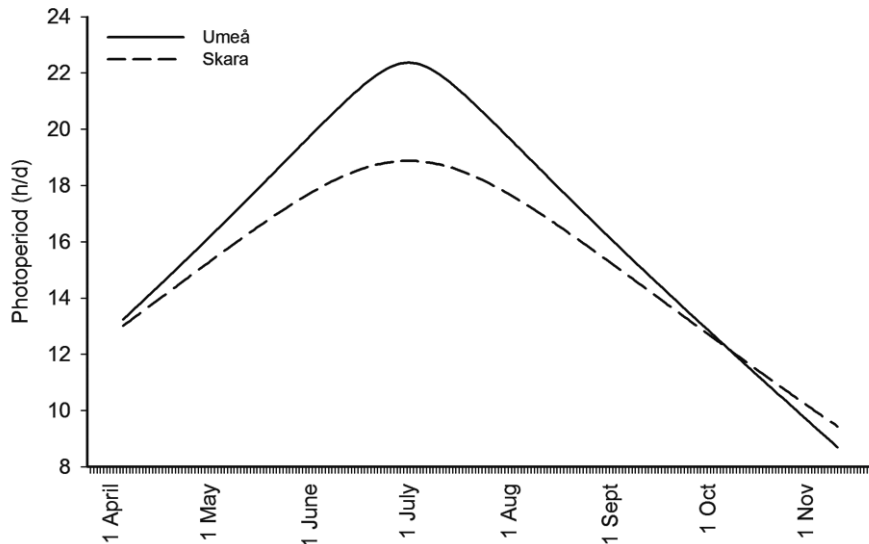
Figure 9. Photoperiod (hours day^{-1}) at Umeå and Skara, Sweden during the growing season.

Growth rate

Differences in the growth rate of forages grown at the different sites could be another reason for the observed differences in α-tocopherol and β-carotene concentration. A high growth rate has been related to lower α-tocopherol and β-carotene concentrations in forages (Booth & Hobson-Frohock, 1961; Booth, 1964; Livingston *et al.*, 1968).

The growing season started earlier at Skara (26 March and 2 April in 2005 and 2007, respectively) than at Umeå (10 May and 28 April 2005 and 2006, respectively) and the number of days from the start of the growing season to the early cut were fewer at Umeå than at Skara. This indicates that the growth

rate was higher at Umeå, especially since the developmental stage of most species was more advanced at Umeå than at Skara in 2005.

Developmental stages

All forage species, except birdsfoot trefoil seemed to have reached a more advanced developmental stage at Umeå in 2005 than at Skara in 2005. However, the legumes at Umeå 2005 also had a larger proportion of less developed plants than those at Skara in 2005. Advanced maturity has been shown to decrease α-tocopherol concentrations in forages (Brown, 1953), but according to a review by Ballet *et al.* (2000), α-tocopherol and β-carotene concentrations in forage increase until the flowering stage is reached. Livingston *et al.* (1968) found that carotene content in leaves reached a maximum at the beginning of the flowering stage during spring growth and then decreased as flowering progressed. The variation in developmental stages between the plots may be a reason for the inconsistent results.

Leaf blade proportion

The relationship between leaf blade proportion and α-tocopherol and β-carotene concentration in forages was inconsistent between sites. Proportion of leaves is believed to have a large impact on the α-tocopherol and β-carotene concentrations in forage, as these compounds are mainly found in the leaves (Brown, 1953; Park *et al.*, 1983; Ballet *et al.*, 2000). However, leaf blade proportion only differed between meadow fescue grown at Skara and Umeå in 2005 there were no differences in leaf blade proportion in timothy between the sites.

No significant differences in leaf blade proportion of legumes were found between Umeå 2005 and Skara 2005. Lack of difference in leaf blade proportion in birdsfoot trefoil between the sites disagree with the review by Ballet *et al.* (2000) concluding that leaf proportion is the major factor affecting α-tocopherol and β-carotene concentrations of plants. The results are more in agreement with conclusions from the review by Noizere *et al.* (2006) suggesting that leaf proportion has a minor effect on β-carotene concentrations in forages.

5.1.2 Year

Differences between years at the sites were only compared statistically for the forages grown at Skara in 2005 and 2007 as these were both first-year leys.

Skara 2005 vs. 2007

No differences in α-tocopherol concentration of the legumes were found between years at Skara, which could be related to similar developmental stages between the years. β-Carotene concentrations differed more than α-tocopherol concentrations in both grasses and legumes between years at Skara, indicating that β-carotene is more sensitive to small changes in climate and developmental stages than α-tocopherol.

The higher α-tocopherol concentration in meadow fescue at Skara in 2005 than in 2007 could depend on the earlier maturity stage and higher leaf blade proportion of meadow fescue in 2005 than in 2007. The α-tocopherol concentration in meadow fescue was also more similar between cuts at Skara in 2005 than in 2007, which could be due to the smaller variation in maturity of meadow fescue between cuts in 2005. This finding agrees with Brown (1953) and suggests that, at least in grasses, α-tocopherol concentration decreases with maturity. However, inconsistent differences in α-tocopherol concentrations in grasses between cuts at Umeå in 2006 did not support this suggestion.

Umeå 2005 vs. 2006

Averaged over cuts, legumes had higher α-tocopherol concentrations at Umeå in 2006 than in 2005 although leaf blade proportions seemed similar between these years. Forages in Umeå 2005 had generally reached a later developmental stage at the cutting time than forages cut at corresponding cutting occasions in 2006. None of the legumes had, however, reached the beginning of flowering stage. The β-carotene concentrations behaved differently to the α-tocopherol concentrations, being numerically higher in 2005 than in 2006.

Small and inconsistent numerical differences in α-tocopherol concentrations in grasses averaged over cuts were found between years, although leaf blade proportion was numerically higher in all grass species and cuts at Umeå in 2006 compared with 2005. β-Carotene concentrations in the grasses were higher in 2006 than in 2005. Light intensity and temperature were similar between years during the cuts.

Denmark 2005 vs. 2006

The most noticeable numerical difference in α-tocopherol and β-carotene concentration between years in Denmark was found in the fourth harvest, where forage harvested in 2005 had higher vitamin concentrations than forage harvested in 2006. Differences in β-carotene concentration were more pronounced, but α-tocopherol concentration was also numerically higher in most grass and legume species in the fourth harvest in 2005 compared with

2006. This might be an effect of a higher growth rate between harvests in 2006 than in 2005, but could also be an effect of later harvest date in 2005 compared with 2006.

5.1.3 Species

Legumes

Birdsfoot trefoil had higher α-tocopherol and β-carotene concentrations than clovers for most cuts and sites, with the differences being more pronounced in α-tocopherol than β-carotene concentration.

The higher vitamin concentrations of birdsfoot trefoil compared with red clover could occasionally be explained by higher leaf blade proportion of the birdsfoot trefoil. However, as mentioned earlier, leaf blade proportion could not explain all the differences in vitamin concentration between species.

When exposed to high light intensities, α-tocopherol and β-carotene synthesis accumulates in stressed plants to control production of ROS (Munné-Bosch & Alegre, 2002; Holländer-Czytko *et al*., 2005; Munné-Bosch, 2005). The differences in α-tocopherol and β-carotene concentration in different species could thus be related to differences stress tolerance between species.

Differences in stress tolerance between plants could also explain the lack of difference in vitamin concentrations in red clover between Umeå and Skara in 2005, as the red clover cultivars were chosen based on adaptation to the sites.

The shorter vegetative period of birdsfoot trefoil than of red clover (Barnes *et al*., 1995) might result in a larger proportion of older leaves that are less actively growing than in red clover. Ageing leaves and less actively growing leaves have been shown to have a higher α-tocopherol concentration than younger and actively growing leaves (Booth & Hobson-Frohock, 1961; Booth, 1964; Tramontano *et al*., 1992; Holländer-Czytko *et al*., 2005). At Skara, birdsfoot trefoil was in later developmental stage than red clover, whereas the opposite was seen at Umeå.

White clover and red clover grown in Denmark had similar vitamin concentrations with small and inconsistent differences between species, within harvests. Averaged over harvests and years, white clover had numerically higher α-tocopherol and β-carotene concentrations than red clover. This was in agreement with results by Søegaard *et al*. (2010).

Grasses

Meadow fescue had higher α-tocopherol and β-carotene concentration than timothy at Skara in 2005 when averaged over cuts, and had also a higher leaf blade proportion than timothy. This supports earlier reports that leaf blade

proportion has a large effect on vitamin concentrations in forage (Brown, 1953). However, at Umeå in 2006 leaf blade proportion was higher in meadow fescue than in timothy, averaged over cuts, whereas timothy grown with birdsfoot trefoil had higher α-tocopherol concentration than meadow fescue, when averaged over cuts.

Leaf blade proportion of the forages was not determined in Denmark and no clear differences in α-tocopherol and β-carotene concentrations were found between timothy and perennial ryegrass in Denmark.

5.1.4 Cutting date and season

Cutting date had little effect on vitamin concentrations in grasses and legumes at Umeå even though developmental stage seemed to increase more at Umeå than Skara from early to late cut. Concentrations of α-tocopherol and β-carotene are believed to increase in the early developmental stages of plants and then decrease when the ROS accumulation is higher than the production of antioxidants (Munné-Bosch & Alegre, 2002; Munné-Bosch, 2005). The production of ROS might also be affected by photoperiod, as higher vitamin concentrations have been found in shaded swards than in un-shaded (Olsson *et al.*, 1955; Hjarde *et al.*, 1963). The ROS production might then already have been higher at Umeå than at Skara, which might have resulted in smaller decrease in vitamin concentrations with delayed cutting date at Umeå. The lack of decrease of α-tocopherol and β-carotene concentrations in red clover in Skara could be related to the small differences in developmental stages and, thus, small differences in production and consumption of antioxidants in the plant.

α-Tocopherol and β-carotene concentrations in the forage between harvests were not compared statistically in Denmark as harvest dates and weeks between harvests differed. However, as mentioned earlier there was a numerical increase in vitamins in the fourth harvest in 2005 compared with the earlier harvests. A similar but less evident increase was observed in 2006. Possible explanations for the higher vitamin concentrations of the forage in the fourth harvest compared with earlier harvests are the lower temperature and shorter photoperiod. It could be an effect of growth rate, suggesting a slower growth rate between the third and fourth harvests in 2005 than between the other harvests.

Previous studies reported that the growth rate of plants affects vitamin concentration and that during periods of higher growth rate, plants have a lower vitamin concentration (Booth & Hobson-Frohock, 1961; Booth, 1964). Seasonal effects on α-tocopherol and β-carotene concentrations of forages have been reported with higher concentration in the autumn and winter months

(Booth & Hobson-Frohock, 1961; Livingston *et al.*, 1968; Arizmendi-Maldonado *et al.*, 2003).

5.1.5 Conservation

Weather conditions were good and wilting time was short, which probably had a positive effect on the vitamin losses during wilting. Wilting conditions are known to affect vitamin concentrations of forage (Carter, 1960).

Silages were well fermented and losses of α-tocopherol concentration during ensiling were only observed in acid-treated red clover mixtures. α-Tocopherol concentration actually increased during ensiling of the Bft+Ti mixture in all silage treatments. This supports earlier findings that ensiling is a good method to preserve vitamins in forages and that losses of α-tocopherol and β-carotene concentrations during ensiling could be species dependent (Mogensen *et al.,* In press.; Ballet *et al.*, 2000; Beeckman *et al.*, 2010).

The higher α-tocopherol concentrations in inoculant-treated silages compared with control silages of red clover indicate that bacterial inoculants have a positive influence on the α-tocopherol concentration of silage. Bacterial inoculation might be a good means of introducing α-tocopherol producing bacteria (Tani & Osuka, 1989; Tani & Tsumura, 1989).

5.2 Dairy cows, Paper IV

5.2.1 α-Tocopherol, β-carotene and retinol concentrations in blood

α-Tocopherol

Cows supplemented with a high dose of RRR α-tocopheryl acetate around calving tended to have higher α-tocopherol concentrations in blood plasma than C cows at calving. At three weeks PC, the difference was significant. Similar results have been found in previous studies (Meglia *et al.*, 2006; Weiss *et al.*, 2009). Blood plasma levels were below the desired level of 3 mg L^{-1} (Weiss, 1999) in both treatments at calving. However, three weeks PC α-tocopherol concentrations were above 3 mg L^{-1} in both treatment groups in both years with higher α-tocopherol concentrations in the blood plasma of E cows compared with C cows.

Later in lactation, the α-tocopherol concentration of blood plasma did not differ between treatment groups and levels were well above 3 mg L^{-1} in both groups in both years. The similar α-tocopherol concentrations later in lactation in Exp 1, when none of the groups was supplemented with synthetic vitamin E, compared with Exp 2, when all cows were supplemented according to Swedish recommendations, indicate that duration of vitamin E supplementation could

be restricted to only include the transition period. However, α-tocopherol concentrations in the silage and, thus, intake of basal α-tocopherol from the feed differed in Exp 1 and 2 and more experiments are needed before such conclusions can be confirmed.

β-Carotene and retinol

The adequate levels of β-carotene and retinol in blood plasma in late lactation in Exp 1 suggest that the β-carotene levels in the forage were high enough to support cows with sufficient amounts of β-carotene and retinol later in lactation. However, retinol levels at calving in Exp 1 were deficient (< 1.5 mg L^{-1}), indicating that vitamin A should also be supplemented to dairy cows at least during the transition period around calving.

Retinol levels of 3 mg L^{-1} is regarded as sufficient (Herdt & Stowe, 1991) and according to the conversion factor of 400 mg carotene IU^{-1} of vitamin A all cows received sufficient amounts of β-carotene to cover requirements of vitamin A and, thus, retinol. However, the conversion factor has been questioned and high concentrations of β-carotene in the diet are known to decrease both the conversion factor and the absorption of β-carotene (McDowell, 2000).

The lower blood plasma concentration of β-carotene and the tendency for lower retinol concentration of blood plasma at calving in the E cows in Exp 1 could be an effect of competitiveness in uptake of α-tocopherol and β-carotene. This has been reported earlier (Weiss, 1994) and was seen on one sampling occasion in Paper IV. In Exp 1, β-carotene concentration was below the recommended level of 3 mg L^{-1} in plasma in E cows at calving and in C cows three weeks PC.

5.2.2 α-Tocopherol, β-carotene and retinol concentrations in milk

α-Tocopherol

The lack of treatment effect on α-tocopherol concentration in milk in Exp 1, in contrast to the differences found in Exp 2, could be an effect of the RRR α-tocopherol concentrations in the basal diet offered to cows in Exp 1 compared with Exp 2.

Cows in Exp 1 and 2 were offered similar amounts of IU of vitamin E and the absorption of α-tocopherol was probably similar in both years as cows do not discriminate between α-tocopherol forms in the intestine (Rébeillé & Douce, 2011b). However, even though blood plasma concentrations of α-tocopherol were similar between years, the ratio of the RRR form to the seven other stereoisomers in the blood probably differed between experiments. The

retention time of the 2R and 2S isomers differs in tissues and cows offered *all-rac* α-tocopheryl acetate usually have smaller proportions of the RRR forms circulating in the blood compared with cows offered only the RRR form (Meglia *et al*., 2006).

Cows in Exp 1 probably had sufficient amounts of the 2R forms circulating in the blood plasma to excrete maximal amounts of α-tocopherol to the milk in both treatment groups. Control cows in Exp 2 most likely had more 2S forms circulating in the body and as only small amounts of the 2S stereoisomers are transferred to milk (Meglia *et al*., 2006; Weiss *et al*., 2009), lower α-tocopherol concentrations were found in milk from C cows than from E cows in Exp 2.

Cows in Exp 1 had numerically higher concentrations of α-tocopherol in their milk both three weeks PC and later in lactation than cows in Exp 2. This supports the claim in a previous study (Agabriel *et al*., 2007) that concentration of α-tocopherol in silage has a large effect on α-tocopherol concentration in milk.

β-Carotene and retinol

The E cows in Exp 2 had higher concentrations of β-carotene and retinol in the milk than C cows. This could be a complementary anti-oxidative effect caused by the higher α-tocopherol concentration of the milk. α-Tocopherol and β-carotene are both antioxidants and could cover for each other (Munné-Bosch, 2005). Noiziere *et al*. (2006) concluded in a review that the nature and amount of forage consumed by dairy cows is the main factor controlling carotenoid and retinol concentrations in dairy products.

6   Conclusions

Forage plants grown in southern Sweden generally had higher vitamin concentrations than forage plants grown in the north of the country. Smaller differences were found between years.

Of the legumes, birdsfoot trefoil had higher vitamin concentrations than clovers in most trials, but it could not be concluded whether this was an effect of differences in leaf blade proportion, developmental stage or stress tolerance.

More inconsistent results were found regarding differences in vitamin concentrations between grass species and none of the grasses could be concluded to have consistently higher vitamin concentration than the others.

Cutting date had a larger effect on vitamin concentrations in forage species at Skara than at Umeå, but the differences could not be related to differences in developmental stages or leaf blade proportion between cuts.

The vitamins in the forage mixtures were well preserved during wilting and ensiling and no losses of α-tocopherol or β-carotene were found during ensiling of the untreated forage. Use of an acid decreased α-tocopherol concentration of the red clover mixtures during ensiling, whereas inoculated silage had higher α-tocopherol concentrations than control silage of the red clover mixtures. Mixtures of birdsfoot trefoil increased α-tocopherol concentration during ensiling regardless of treatment. Silages with birdsfoot trefoil had higher vitamin concentration than silages with red clover in all treatments.

A high daily dose of semi-natural vitamin around calving increased the α-tocopherol concentration in the blood plasma when the supplement was offered. However, it had no effect on vitamin concentrations in the milk when the cows already had high concentrations of natural α-tocopherol and β-carotene from feeds in the basal diet. Supplementation with the semi-natural form of α-tocopherol had no effect on vitamin concentrations in the blood plasma later in lactation, when both treatment groups had adequate concentrations of α-tocopherol, β-carotene and retinol.

7   Future Perspectives

An increased vitamin concentration in forages would be beneficial for dairy cows, calves and humans as the natural α-tocopherol form has a higher bioavailability than the synthetic form. It would be interesting to compare vitamin concentrations in additional forage species in order to devise mixtures with a potential for high vitamin concentrations. It would also be interesting to feed those forage species or mixtures to dairy cows to determine availability in the cow.

The higher vitamin concentrations in milk from cows fed silage with higher vitamin concentration could have a large impact of calf health. Calves are born with small reserves of α-tocopherol and β-carotene and are dependent on high concentrations in the colostrum to increase plasma levels of the vitamins. Feeding silages with higher α-tocopherol and β-carotene concentrations would, thus, increase levels of α-tocopherol and β-carotene in the plasma of calves and might result in lower calf mortality.

The effect of ensiling and additive was remarkable and more studies should be carried out to determine why silage treated with the bacterial inoculant had higher α-tocopherol concentration than the wilted birdsfoot trefoil mixture and the control and acid-treated silages of red clover. The bacterial inoculant resembles media used to increase α-tocopherol production by the cyanobacteria *Euglena gracilis* Z. It would be interesting to examine whether these or other cyanobacteria are present on plants, whether they can produce α-tocopherol during ensiling and whether such production is large enough to make a difference.   .

8 Populärvetenskaplig sammanfattning

Vitaminer är livsnödvändiga för att kroppen ska fungera och brist på vitaminer leder till nedsatt hälsa och fertilitet och kan vid allvarlig brist leda till döden. Behovet av att få i sig vitaminerna från fodret varierar mellan djur och därför behöver vissa vitaminer tillsättas i större mängder medan andra inte behöver tillsättas alls. Behovet varierar även med djurens ålder, produktion och dräktighet.

Vitaminerna delas upp i fettlösliga vitaminer A, D E och K och vattenlösliga vitaminer B och C. Mjölkkor anses uppfylla sitt behov av vitamin B, C och K eftersom de oftast tillgodoser behovet av dessa vitaminer med hjälp av mikroorganismerna i våmmen. Däremot har man sett fördelar av att tillsätta vitamin A, D och E i foderstaten. Den här avhandlingen fokuserar på α-tokoferol (vitamin E) och β-karotin (pro-vitamin A) eftersom dessa vitaminer kan vara svåra att få i sig i tillräckliga mängder genom det som finns naturligt i fodret.

Anledningen till att vitamin A och E ofta tillsätts är att man vet att korna har stort behov av dessa vitaminer framförallt runt kalvning då korna är utsatta för hög stress när mjölkproduktionen ska komma igång. Kor är mottagligare för sjukdomar i början av laktationen än senare och då är det viktigt att de får i sig tillräckliga mängder α-tokoferol och β-karotin för att få bästa möjliga skydd. Stora mängder av vitaminerna utsöndras till råmjölken och det kan bli ett glapp mellan intag och utsöndring då korna dessutom har ett lågt foderintag i slutet av dräktigheten.

Vitamin A och E tillsätts oftast i syntetisk form eftersom det är enklare och billigare att producera än tillskott av naturligt α-tokoferol och vitamin A. Flera studier har dock visat på fördelar av att tillsätta den naturliga formen av α-tokoferol eftersom den syntetiska har en sämre effekt än den naturliga och därför behöver tillsättas i större mängder.

Ett bra vallfoder har goda förutsättningar att täcka en stor del av kornas vitaminbehov. Det utgör den största källan av naturliga vitaminer i fodret till mjölkkorna. Vallfodrets innehåll av α-tokoferol och β-karotin kan dock variera väldigt mycket beroende på var det odlas, vilka vallarter som används, skördetidpunkt, klimat, konserveringsmetod m.m.

α-Tokoferol och β-karotin bildas i fotosyntetiska organismer som växter och alger. De är antioxidanter och en stor del av deras funktion är att skydda växter och djurs celler från oxidation och skadliga produkter från oxideringsprocessen. β-Karotin har även en viktig roll i att samla solljus till växtens fotosyntes och att ge färg till blad och blommor.

Vallväxter som odlas i södra Sverige har ofta ett högre vitamininnehåll än vallväxter som odlas i norra Sverige. Detta kan bero på en mängd faktorer och några av dem kan vara skillnader i tillväxthastighet, temperatur och solinstrålning. Det kan också bero på att växterna skördats vid olika mognadsstadier. Vilka vallväxter som ingår i vallfröblandningen har också betydelse. Bladrika vallväxter har ofta ett högre vitamininnehåll än vallväxter med mindre andel blad. En stor del av det α-tokoferol och β-karotin som finns i vallväxterna finns nämligen i bladen. Tillväxthastighet och stresskänslighet hos vallväxterna påverkar också deras innehåll av α-tokoferol och β-karotin.

Skördetidpunkt och utvecklingsstadium av växterna har också effekt på vitamininnehållet och man har sett att vitaminkoncentrationen i växterna kan variera beroende på om skörden är tagen på våren, sommaren eller hösten. Variationen i α-tokoferol- och β-karotininnehåll vid olika utvecklingsstadier är mycket stor och det kan bero på både svårigheter att bedöma utvecklingsstadium men också på klimateffekter under växtperioden. Vissa hävdar att vitamininnehållet är högst i unga växter i det vegetativa stadiet medan andra hävdar att vitamininnehållet i växterna ökar fram till att växten börjar blomma för att sedan minska.

Förhållandena vid skörd, konservering och utfodring är ytterligare faktorer som påverkar vitamininnehållet i vallfodret. Ensilage innehåller ofta betydligt mer vitaminer än t ex hö. Faktorer som kan påverka vitaminförluster vid ensilering är väderleksförhållande under förtorkning, ensileringsmetod, ensileringsmedel och lagringstid.

Syftet med vallstudierna i den här avhandlingen var att se hur α-tokoferol- och β-karotinkoncentrationerna varierar i vallväxterna käringtand, rödklöver, vitklöver, timotej, ängssvingel och engelskt rajgräs odlade på olika orter och skördade vid olika mognadsstadier och tidpunkt på säsongen. Olika ensileringsmedel och vallväxtblandningars effekt på vitamininnehåll och förluster under ensilering studerades också. Syftet med mjölkkoförsöket var att se om E och A vitamin behöver tillsättas under hela laktationen till mjölkkor då

det finns ett önskemål inom ekologisk produktion att begränsa tillskottsutfodring. Ett annat syfte var att se vilka effekter ett större dagligt tillskott av den naturliga formen av α-tokoferol skulle ha vid utfodring under tre veckor före beräknad kalvning till tre veckor efter kalvning.

Studierna bekräftade att det är en stor variation i vitamininnehållet i vallväxterna. Inga renodlade slutsatser kunde dras eftersom det är så många faktorer som påverkar vitamininnehållet i vallfodret. Käringtand, timotej och ängssvingel odlade i Umeå hade lägre vitamininnehåll än samma växter odlade i Skara men det gick inte att säga vad som påverkade detta mest. Vitaminkoncentrationerna i rödklöver skiljde sig inte mellan orterna, vilket kan bero på att olika sorter av rödklöver odlades i Umeå och Skara medan samma sorter av de andra arterna odlades på båda orter. Vallväxterna i Skara och Umeå skördades vid tre olika tillfällen i första skörden med en veckas mellanrum, men det var främst i Skara som vitamininnehållet minskade med senare skörd. Det kan ha varit en effekt av variationen i utvecklingsstadiet men det kan också ha berott på att växterna i Skara hade högre vitaminkoncentrationer från början och att minskningen därför var tydligare där.

Käringtand hade högre vitamininnehåll än klöver i de flesta studierna medan det var mindre skillnad mellan röd- och vitklöver. Vitklöver hade lite högre vitamininnehåll än rödklöver i några av skördarna men inte i alla. Det var svårare att hitta en "vinnare" bland gräsen där det varierade mer mellan skördar och orter vilka gräs som hade högst vitamininnehåll.

Ett högre vitamininnehåll i den sent skördade fjärdeskörden i en av studierna kan tyda på att växterna har ett högre vitamininnehåll senare på hösten än under vår och sommar men fler studier behöver göras för att riktiga slutsatser ska kunna dras.

Under ensileringsförsöket jämfördes tre blandningar; käringtand+timotej, rödklöver+timotej och rödklöver+ängssvingel. Det var ingen skillnad mellan blandningarna i det nyslagna materialet men under ensileringen noterades vissa skillnader, vilka tyder på att olika blandningar har olika förutsättningar att behålla vitaminnivåer i grönmassan under ensileringen. I blandningen med käringtand ökade innehållet av vitamin E under ensileringen vilket är anmärkningsvärt. Två ensileringsmedel (syra och bakteriepreparat) jämfördes mot en kontroll utan ensileringsmedel där användningen av ett bakteriepreparat hade positiv inverkan på E vitamin under ensilering. Rödklöverblandningarna som behandlades med bakteriepreparatet hade högre E vitamininnehåll än obehandlade rödklöverensilage. Tillsats av syran minskade innehållet av E vitamin under ensilering av rödklöverblandningarna, medan det ökade i käringtandblandningen.

Vitaminstudien på mjölkkor visade en positiv effekt av att ge ett större vitamintillskott till korna under den känsliga perioden runt kalvning. Det är dock oklart om det räcker med enbart α-tokoferoltillskott eller om även β-karotin eller A vitamin bör tillsättas. Resultaten från studien stödjer påståenden från tidigare studier som säger att vallfodret har stor betydelse för vitaminförsörjning av mjölkkor. Resultaten visar på fördelar med att ha ett vallfoder med höga vitaminnivåer då kor som utfodrades med ett ensilage med högre vitaminkoncentrationer verkade ha högre koncentration av α-tokoferol i mjölken än kor som utfodrades med ett vallfoder med lägre vitaminkoncentrationer.

References

Agabriel, C., Cornu, A., Journal, C., Sibra, C., Grolier, P. & Martin, B. (2007). Tanker milk variability according to farm feeding practices: Vitamins A and E, carotenoids, color, and terpenoids. *Journal of Dairy Science* 90(10), 4884-4896.

Arizmendi-Maldonado, D., McDowell, L.R., Sinclair, T.R., Mislevy, P., Martin, F.G. & Wilkinson, N.S. (2003). Alpha-tocopherol and beta-carotene concentrations in tropical grasses as affected by increasing daylength. *Communications in Soil Science and Plant Analysis* 34(3-4), 519-530.

Asensi-Fabado, M.A. & Munné-Bosch, S. (2010). Vitamins in plants: occurrence, biosynthesis and antioxidant function. *Trends in Plant Science* 15(10), 582-592.

Baldi, A., Savoini, G., Pinotti, L., Monfardini, E., Cheli, F. & Dell'Orto, V. (2000). Effects of vitamin E and different energy sources on vitamin E status, milk quality and reproduction in transition cows. *Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine* 47(10), 599-608.

Ballet, N., Robert, J.C. & Williams, P.E.V. (2000). *Vitamins in forages*. Wallingford UK: CABI Publishing. Forage evaluation in ruminant nutrition.

Barnes, R.F., Miller, D.A. & Nelson, C.J. Eds. (1995). *Forages – An introduction to grassland agriculture* Ames, Iowa: Iowa State University Press; 1.

Beeckman, A., Vicca, J., Van Ranst, G., Janssens, G.P.J. & Fievez, V. (2010). Monitoring of vitamin E status of dry, early and mid-late lactating organic dairy cows fed conserved roughages during the indoor period and factors influencing forage vitamin E levels. *Journal of Animal Physiology and Animal Nutrition* 94(6), 736-746.

Blatt, D.H., Pryor, W.A., Mata, J.E. & Rodriguez-Proteau, R. (2004). Re-evaluation of the relative potency of synthetic and natural alpha-tocopherol: experimental and clinical observations. *Journal of Nutritional Biochemistry* 15(7), 380-395.

Booth, V.H. (1964). Alpha-tocopherol content of forage crops. *Journal of the Science of Food and Agriculture* 15(5), 342-344.

Booth, V.H. & Hobson-Frohock, A. (1961). Alpha-tocopherol content of leaves as affected by growth rate. *Journal of the Science of Food and Agriculture* 12(3), 251-255.

Bouwstra, R.J., Nielen, M., Stegeman, J.A., Dobbelaar, P., Newbold, J.R., Jansen, E.H.J.M. & Werven, T.V. (2010). Vitamin E supplementation during the dry period in dairy cattle. Part I:

Adverse effect on incidence of mastitis postpartum in a double-blind randomized field trial. *Journal of Dairy Science* 93(12), 5684-5695.

Bramley, P.M., Elmadfa, I., Kafatos, A., Kelly, F.J., Manios, Y., Roxborough, H.E., Schuch, W., Sheehy, P.J.A. & Wagner, K.H. (2000). Vitamin E. *Journal of the Science of Food and Agriculture* 80(7), 913-938.

Brigelius-Flohe, R. (2006). Bioactivity of vitamin E. *Nutrition Research Reviews* 19(2), 174-186.

Brigelius-Flohe, R. & Traber, M.G. (1999). Vitamin E: function and metabolism. *FASEB Journal* 13(10), 1145-1155.

Brown, F. (1953). The tocopherol content of farm feeding-stuffs. *Journal of the Science of Food and Agriculture* 4(4), 161-165.

Campbell, M.H. & Miller, J.K. (1998). Effect of supplemental dietary vitamin E and zinc on reproductive performance of dairy cows and heifers fed excess iron. *Journal of Dairy Science* 81(10), 2693-9.

Carter, W.R.B. (1960). A review of nutrient losses and efficiency of conserving herbage as silage, barn-dried hay and field-cured hay. *Journal of the British Grassland Society* 15(3), 220-230.

DellaPenna, D. (2005). A decade of progress in understanding vitamin E synthesis in plants. *Journal of Plant Physiology* 162(7), 729-737.

DellaPenna, D. & Pogson, B.J. (2006). Vitamin synthesis in plants: tocopherols and carotenoids. In: *Annual Review of Plant Biology*. pp. 711-738.

Dersjant-Li, Y. & Peisker, M. (2010). A critical review of methodologies used in determination of relative bio-availability ratio of RRR-alpha-tocopheryl acetate and all-rac-alpha-tocopheryl acetate. *Journal of the Science of Food and Agriculture* 90(10), 1571-1577.

Drackley, J.K. (1999). Biology of dairy cows during the transition period: the final frontier? *Journal of Dairy Science* 82(11), 2259-2273.

Edmonson, A.J., Lean, I.J., Weaver, L.D., Farver, T. & Webster, G. (1989). A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science* 72(1), 68-78.

EU (1999). Council Directive no. 1804/1999. *Official Journal of European Communities L222/1.*

Falk, J. & Munne-Bosch, S. (2010). Tocochromanol functions in plants: antioxidation and beyond. *Journal of Experimental Botany* 61(6), 1549-1566.

Fraser, P.D. & Bramley, P.M. (2004). The biosynthesis and nutritional uses of carotenoids. *Progress in Lipid Research* 43(3), 228-265.

Frye, T.M., Williams, S.N. & Graham, T.W. (1991). Vitamin deficiencies in cattle. *Veterinary Clinics of North America-Food Animal Practice* 7(1), 217-275.

Goff, J.P. & Stabel, J.R. (1990). Decreased plasma retinol, alpha-tocopherol, and zinc concentration during the periparturient period: effect of milk fever. *Journal of Dairy Science* 73(11), 3195-3199.

Gustavsson, A.M. (2011). A developmental scale for perennial forage grasses based on the decimal code framework. *Grass and Forage Science* 66(1), 93-108.

Harrison, E.H. (2012). Mechanisms involved in the intestinal absorption of dietary vitamin A and pro-vitamin A carotenoids. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* 1821(1), 70-77.

Havaux, M., Eymery, F., Porfirova, S., Rey, P. & Dormann, P. (2005). Vitamin E protects against photoinhibition and photooxidative stress in Arabidopsis thaliana. *Plant Cell* 17(12), 3451-3469.

Herdt, T.H. & Stowe, H.D. (1991). Fat-soluble vitamin nutrition for dairy cattle. *Veterinary Clinics of North America-Food Animal Practice* 7(2), 391-415.

Hirschberg, J. (1999). Production of high-value compounds: carotenoids and vitamin E. *Current Opinion in Biotechnology* 10(2), 186-191.

Hjarde, W., Hellstrom, V. & Akerberg, E. (1963). The contents of tocopherol and carotene in red clover as dependent on variety, conditions of cultivation and stage of development. *Acta Agriculturae Scandinavica* 13(1), 3-16.

Holländer-Czytko, H., Grabowski, J., Sandorf, I., Weckermann, K. & Weiler, E.W. (2005). Tocopherol content and activities of tyrosine aminotransferase and cystine lyase in Arabidopsis under stress conditions. *Journal of Plant Physiology* 162(7), 767-770.

Horn, M., Gunn, P., Van Emon, M., Lemenager, R., Burgess, J., Pyatt, N.A. & Lake, S.L. (2010). Effects of natural (RRR alpha-tocopherol acetate) or synthetic (*all-rac* alpha-tocopherol acetate) vitamin E supplementation on reproductive efficiency in beef cows. *Journal of Animal Science* 88(9), 3121-3127.

Hymøller, L., Jensen, S.K., Lindqvist, H., Johansson, B., Nielsen, M.O. & Nadeau, E. (2009). Supplementing dairy steers and organically managed dairy cows with synthetic vitamin D_3 is unnecessary at pasture during exposure to summer sunlight. *Journal of Dairy Research* 76(3), 372-378.

Jensen, S.K. (1994). Retinol determination in milk by hplc and fluorescence detection. *Journal of Dairy Research* 61(2), 233-240.

Jensen, S.K., Jensen, C., Jakobsen, K., Engberg, R.M., Andersen, J.O., Lauridsen, C., Sorensen, P., Skibsted, L.H. & Bertelsen, G. (1998). Supplementation of broiler diets with retinol acetate, beta-carotene or canthaxanthin: Effect on vitamin status and oxidative status of broilers in vivo and on meat stability. *Acta Agriculturae Scandinavica Section A-Animal Science* 48(1), 28-37.

Jensen, S.K., Johannsen, A.K.B. & Hermansen, J.E. (1999). Quantitative secretion and maximal secretion capacity of retinol, beta-carotene and alpha-tocopherol into cows' milk. *Journal of Dairy Research* 66(4), 511-522.

Jensen, S.K. & Lauridsen, C. (2007). α-Tocopherol Stereoisomers. In: Gerald, L. (Ed.) *Vitamins & Hormones*. pp. 281-308 Academic Press; Volume 76.

Jensen, S.K. & Nielsen, K.N. (1996). Tocopherols, retinol, beta-carotene and fatty acids in fat globule membrane and fat globule core in cows' milk. *Journal of Dairy Research* 63(4), 565-574.

Kalac, P. (1983). Losses of beta-carotene in unwilted forage crops during silage-making and feeding. *Animal Feed Science and Technology* 9(1), 63-69.

Kalac, P. & Kyzlink, V. (1979). Losses of beta-carotene in red-clover in an acid-medium during ensiling. *Animal Feed Science and Technology* 4(2), 81-89.

Kume, S. & Toharmat, T. (2001). Effect of colostral β-carotene and vitamin A on vitamin and health status of newborn calves. *Livestock Production Science* 68(1), 61-65.

Livingston, A., Smith, D., Carnahan, H.L., Knowles, R.E., Nelson, J.W. & Kohler, G.O. (1968). Variation in xanthophyll and carotene content of lucerne clovers and grasses. *Journal of the Science of Food and Agriculture* 19(11), 632-636.

McDowell, L.R. (2000). *Vitamins in animal and human nutrition*. Ames: Iowa State University Press.

Meglia, G.E., Jensen, S.K., Lauridsen, C. & Waller, K.P. (2006). Alpha-tocopherol concentration and stereoisomer composition in plasma and milk from dairy cows fed natural or synthetic vitamin E around calving. *Journal of Dairy Research* 73(2), 227-234.

Michal, J.J., Heirman, L.R., Wong, T.S., Chew, B.P., Frigg, M. & Volker, L. (1994). Modulatory effects of dietary β-carotene on blood and mammary leukocyte function in periparturient dairy cows. *Journal of Dairy Science* 77(5), 1408-1421.

Mogensen, L., Kristensen, T., Søegaard, K., Jensen, S.K. & Sehested, J. Alfa-tocopherol and beta-carotene in roughages and milk in organic dairy herds. *Livestock Science* (In press).

Munné-Bosch, S. (2005). The role of alpha-tocopherol in plant stress tolerance. *Journal of Plant Physiology* 162(7), 743-748.

Munné-Bosch, S. & Alegre, L. (2002). The function of tocopherols and tocotrienols in plants. *Critical Reviews in Plant Sciences* 21(1), 31-57.

Nadeau, E., Englund, J.E. & Gustafsson, A.H. (2007). Nitrogen efficiency of dairy cows as affected by diet and milk yield. *Livestock Science* 111(1-2), 45-56.

Nadeau, E., Johansson, B., Jensen, S.K. & Olsson, G. (2004). Vitamin content of forages as influenced by harvest and ensiling techniques. *Land use systems in grassland dominated regions. Proceedings of the 20th General Meeting of the European Grassland Federation, Luzern, Switzerland, 21-24 June 2004*, 891-893.

Noziere, P., Graulet, B., Lucas, A., Martin, B., Grolier, P. & Doreau, M. (2006). Carotenoids for ruminants: From forages to dairy products. *Animal Feed Science and Technology* 131(3-4), 418-450.

NRC (2001). *Nutrient requirements of dairy cattle.* 7th rev. edition. ed. Washington, DC. National Academy Press.

Olsson, N., Akerberg, E. & Blixt, B. (1955). Investigations concerning formation, preservation and utilization of carotene. *Acta Agriculturae Scandinavica* 5, 113-184.

Park, Y.W., Anderson, M.J., Walters, J.L. & Mahoney, A.W. (1983). Effects of processing methods and agronomic variables on carotene contents in forages and predicting carotene in alfalfa hay with near-infrared-reflectance spectroscopy. *Journal of Dairy Science* 66(2), 235-245.

Persson Waller, K., Sandgren, C.H., Emanuelson, U. & Jensen, S.K. (2007). Supplementation of RRR-alpha-tocopheryl acetate to periparturient dairy cows in commercial herds with high mastitis incidence. *Journal of Dairy Science* 90(8), 3640-3646.

Politis, I., Bizelis, I., Tsiaras, A. & Baldi, A. (2004). Effect of vitamin E supplementation on neutrophil function, milk composition and plasmin activity in dairy cows in a commercial herd. *Journal of Dairy Research* 71(3), 273-278.

Rébeillé, F. & Douce, R. (2011a). *Biosynthesis of vitamins in plants. Part A, Vitamins A, B1, B2, B3, B5*. Oxford: Academic Press.

Rébeillé, F. & Douce, R. (2011b). *Biosynthesis of vitamins in plants. Part B, Vitamins B6, B8, B9, C, E, K*. Oxford: Academic Press.

SAS (2001). *SAS User's guide*: Statistics. 8.02. SAS Institute Inc., Cary, NC, USA.

Shingfield, K.J., Salo-Vaananen, P., Pahkala, E., Toivonen, V., Jaakkola, S., Piironen, V. & Huhtanen, P. (2005). Effect of forage conservation method, concentrate level and propylene glycol on the fatty acid composition and vitamin content of cows' milk. *Journal of Dairy Research* 72(3), 349-361.

Sniffen, C.J., O'Connor, J.D., Van Soest, P.J., Fox, D.G. & Russell, J.B. (1992). A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *Journal of Animal Science* 70(11), 3562-3577.

Sommer, A. & Davidson, F.R. (2002). Assessment and control of vitamin a deficiency: the annecy accords. *Journal of Nutrition* 132(9), 2845S-2850S.

Spörndly, R. (2003). *Fodertabeller för idisslare 2003*. Uppsala: Sveriges lantbruksuniv. Rapport / Sveriges lantbruksuniversitet, Institutionen för husdjurens utfodring och vård, 0347-9838 ; 257.

Søegaard, K., Jensen, S.K. & Sehested, J. (2010). *Vitaminer, mineraler of foderværdi af græsmarksarter. Økologisk græsmarksproduktion og udnyttelser til mælkeproduktion*: Faculty of Agricultural Sciences, Aarhus University. Internal Report Husbandry, No 27. ISSN 27.

Tani, Y. & Osuka, S. (1989). Alpha-tocopherol production by an analog-resistant strain of Euglena-Gracilis Z. *Agricultural and Biological Chemistry* 53(9), 2313-2318.

Tani, Y. & Tsumura, H. (1989). Screening for tocopherol-producing microorganisms and alpha-tocopherol production by Euglena-Gracilis Z. *Agricultural and Biological Chemistry* 53(2), 305-312.

Traber, M.G. (2000). The bioavailability bugaboo. *The American Journal of Clinical Nutrition* 71(5), 1029-1030.

Tramontano, W.A., Ganci, D., Pennino, M. & Dierenfeld, E.S. (1992). Age-dependent alpha-tocopherol concentrations in leaves of soybean and pinto beans. *Phytochemistry* 31(10), 3349-3351.

Triantaphylidès, C. & Havaux, M. (2009). Singlet oxygen in plants: production, detoxification and signaling. *Trends in Plant Science* 14(4), 219-228.

Vagni, S., Saccone, F., Pinotti, L. & Baldi, A. (2011). Vitamin E bioavailability: past and present insights. *Food and Nutrition Sciences* 2(10), 1088-1096.

Von Lintig, J. (2010). Colors with functions: Elucidating the biochemical and molecular basis of carotenoid metabolism. In: Cousins, R.J. (Ed.) *Annual Review of Nutrition, Vol 30*. pp. 35-56. 978-0-8243-2830-6.

Weiser, H., Riss, G. & Kormann, A.W. (1996). Biodiscrimination of the eight alpha-tocopherol stereoisomers results in preferential accumulation of the four 2R forms in tissues and plasma of rats. *Journal of Nutrition* 126(10), 2539-2549.

Weiser, H. & Vecchi, M. (1981). Stereoisomers of alpha-tocopheryl acetate characterization of the samples by physiochemical methods and determination in the rat resorption-gestation test. *International Journal for Vitamin and Nutrition Research* 51(2), 100-113.

Weiser, H. & Vecchi, M. (1982). Stereoisomers of alpha-tocopheryl acetate II. Biopotencies of all stereoisomers, individually or in mixtures, as determined by rat resorption-gestation tests. *International Journal for Vitamin and Nutrition Research* 52(3), 351-370.

Weiss, W.P. (1999). Vitamin E requirements for protection of dairy cows against infections at parturition. *Special Circular - Ohio Agricultural Research and Development Center* (167), 75-81.

Weiss, W.P., Hogan, J.S., Smith, K.L. & Hoblet, K.H. (1990). Relationships among selenium, vitamin E and mamary-gland health in commercial dairy herds. *Journal of Dairy Science* 73(2), 381-390.

Weiss, W.P., Hogan, J.S., Todhunter, D.A. & Smith, K.L. (1997). Effect of vitamin E supplementation in diets with a low concentration of selenium on mammary gland health of dairy cows. *Journal of Dairy Science* 80(8), 1728-1737.

Weiss, W.P., Hogan, J.S. & Wyatt, D.J. (2009). Relative bioavailability of all-rac and RRR vitamin E based on neutrophil function and total alpha-tocopherol and isomer concentrations in periparturient dairy cows and their calves. *Journal of Dairy Science* 92(2), 720-731.

Weiss, W.P., Hogan, J.S., Smith, K.L., Williams, S.N. (1994). Effect of dietary fat and vitamin E on alfa-tocopherol and beta-carotene in blood of peripartum cows. *Journal of Dairy Science* 77, 1422-1429.

Weissbach, F. A simple method for the correction of fermentation losses measured in laboratory silos. In: Park, R.S*., et al.* (Eds.) *Proceedings of Silage Production and Utilisation*, *Belfast,* July 2005. p. 278: *Proccedings of the 14th International Silage Conference / satelite workshop of the 20th International Grassland Congress.*

Zadoks, J.C., Chang, T.T. & Konzak, C.F. (1974). Decimal code for growth stages of cereals. *Weed Research* 14(6), 415-421.

Acknowledgements

The thesis exists thanks to a great supervisory group who came up with the idea for the included projects and who has supported me throughout these years. First of all I would like to thank my main supervisor **Elisabet Nadeau** for all the time, effort and enthusiasm you have spent on guiding me in a research world of statistics, vitamins, forage etc. You are amazing! **Birgitta Johansson**, you have been a tremendous support and a source of inspiration during the years and have given me a lot of your time and enthusiasm. **Søren Krogh Jensen**, for inviting me to your lab to learn how to analyse α-tocopherol and β-carotene concentrations in feeds, milk and blood and for helping me to focus when my desire to solve all vitamin questions in the world led me onto mysterious paths. **Anne-Maj Gustavsson,** for the large knowledge in plant science and all the support in that field. She has spent a lot of time trying to convince me that a figure says more than thousand words.

I have also been fortunate to cooperate with **Karin Persson Waller**, **Nilla Nilsdotter-Linde** and **Karen Søegaard** in my projects and they have contributed to this thesis by involvement in the planning of the studies and by being co-authors of articles. **Jan-Eric Englund** at SLU in Alnarp has together with Elisabet tried to get me to understand statistics and has helped a lot in performance of the statistics in the papers. Thank you. In the beginning of the

study in Paper IV Tingvall was owned by **Hushållningssällskapet Väst** but in the second year it was bought by **Albert Kupier** and I would really want to thank you and your family for letting us continue with our study, for all the work you spent on our project and for inviting me to your farm and home.

I would like to thank all my **co-workers at HMH**. You are wonderful and you make me smile almost every day. A lot of people have been involved in the performance of the studies. I would like to thank all of you that have helped me with the practical parts of the studies. You have all done a great job!

I also would like to thank

Rolf Tunared at Lanna for ploughing up our plots giving me some extra years to finish this, as I took your advice to make some kids in the mean time seriously.

Karin Wallin and **Frida Dahlström** the party princesses of our section, thank you for making the work "go as a dance" and for all practical work and jokes of course.

Jenny Yngvesson and **Jenny Loberg** for giving me a hug every now and then. I have really appreciated your support.

All the administrative staff and especially **Gudrun Norrman** who has answered my questions about economics, travels, ladok etc.

Gunilla Jacobsson for help during projects and especially for all help finishing this thesis with figures and pictures. We are lucky to have you at the department.

Jan Nilsson for computer support and especially for saving my manuscript when I threw it in the bin by accident. Are you sure that you want to place it in the bin? Yes…

All veterinarians at work, who has treated my hypochondria and answered questions about Ginny's health conditions and for the shots every now and then.

All former and present **PhD-colleges** who have supported and helped me during these years. I would not have made this without your support. **Johanna Wetterlind** for teaching me how to use endnote, for the maps and especially for being such a nice person, **Helene Axelsson**, **Elin Weber** and **Hanna Lomander** for long walks and talks about dogs and other important things in life. I have enjoyed all our PhD-meetings, coffees and lunches. I would especially like to thank all of you that have read and commented on my papers and thesis now in the end. You are fantastic!

Marie Olofsson and the" gang" from Tvååker. Thanks for being such good friends and "partypolare". Even though we don't see each other that often nowadays I always enjoy seeing you and talking to you.

Helen Wikström for sharing my first educational years in agriculture. Munkagårdsskolan wouldn't have been the same without us.

The wonderful, wonderful mum's here in Skara **(Sofia, Cecilia, Hanna, Maria and Maria)** who has made me think about other things than work. I can't express with words how much you mean for me and how I always enjoy meeting you for coffee, chat, shopping, play date or whatever. You make Skara a lovely place.

Oskar Nordbladh for sharing the dream to be a farmer and for being the big brother I always wanted.

Ginny my lovely spotted monster for making me take all those walks and for teaching me that praise and candy can take you a long way.

Lisa Sellberg for your patience with crazy PhD-studying friends (and boyfriend). For your excellent "ordspråk". It's too bad I only could choose one of them.

Emma Brunberg because no one understands me better than you. You always have an answer for all my questions and I love you just as you are.

My little brother **Jonas Danielsson** who probably think I am a little weird and crazy, but, hey we can't all be normal. My **mum** and **dad** for always being there. I hope you know how much I love and appreciate you. You have taught me that nothing is unreachable sometimes you just have to fight a bit harder for it. You are the best parents ever.

Martin my lovely husband who has realized that it is hard to have a smart wife. I hope you know how much you mean to me and how much I value your love and support.

Hampus and **Lova** my wonderful babies (well kids) for being just as you are, alldeles, alldeles underbara.