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25-Hydroxycholecalciferol status in plasma is linearly correlated to daily summer pasture time in cattle at 56°N

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Abstract

In vitro studies with skin samples or pure precursors of cholecalciferol indicated that cholecalciferol synthesis during UV light exposure is a non-linear process. However, *in vitro* studies indicate nothing about the relationship between sunlight exposure and physiological cholecalciferol status of living organisms. Due to the lack of cholecalciferol in plant material, this relationship is important for herbivores including domestic cattle, particularly in organic agriculture, because the use of synthetic additives, like cholecalciferol, is restricted in order to fulfil the principles of sustainable organic production. The major physiological metabolite of cholecalciferol is the liver-derived 25-hydroxycholecalciferol (25(OH)D_3). The purpose of the present study was to determine the relationship between sunlight exposure and 25(OH)D_3 status *in vivo* in large herbivores during mid-summer at 56°N. Five groups of cows were given access to pasture during 15, 30, 75, 150 or 300 min daily for 28 d in June and plasma analysed for 25(OH)D_3. Animals allowed 15, 30 or 75 min of daily access to pasture showed a declining linear relationship between plasma 25(OH)D_3 and sampling day in contrast to animals allowed 150 or 300 min of pasture access which showed linear increasing plasma 25(OH)D_3 status. Determined from the slopes of 25(OH)D_3 concentration curves within treatments, breakeven for maintaining the initial 25(OH)D_3 status of 45 nmol/l was 90 min pasture access per d during summer at 56°N.

Key words: **Cholecalciferol: 25-Hydroxycholecalciferol: Sunlight exposure: Endogenous synthesis: Cattle**

The only source of cholecalciferol (vitamin D_3, D_3) in grazing herbivores is endogenous D_3 produced in the skin during exposure to UV light from sunlight, since common grassland plants do not contain D_3. Endogenous D_3 is derived from 7-dehydrocholesterol (7DHC) produced in epithelial cells from acetate through the cholesterol synthesis pathway[1,2]. Irradiation with UV light between 290 and 315 nm cleaves the C9 and C10 bonds of 7DHC, rendering pre-cholecalciferol (preD_3), which as a result of its thermodynamically unstable *cis–cis* configuration, spontaneously isomerises into D_3 by a rearrangement of its double bonds catalysed by heat at body temperature[2,3], a process completed in 2–4 d[4]. If preD_3 is exposed to excessive amounts of UV light it will, instead of being isomerised into D_3, be turned into different inactive metabolites of D_3 catalysed by UV light[3,5].

Even though the biochemical and physiological processes behind endogenous D_3 synthesis in the skin are well described, it is still heavily debated as to how much UV light or sunlight is necessary to secure and maintain a sufficient D_3 status in plasma, measured as 25-hydroxycholecalciferol (25(OH)D_3), which is the major physiological metabolite of D_3 circulating in plasma and indicative of the physiological D_3 status of living organisms[6]. Solar UV light intensity is affected by latitude, time of day and season; and Engelsen *et al.*[7] showed that cutaneous D_3 synthesis could not be sustained throughout the year at latitudes higher than 50°. In addition, clouds, aerosols and thick ozone events reduce the duration of D_3 synthesis and can induce a 'vitamin D winter' even at the equator[7].

In studies on rat and pig skin subjected to UV light, between 15 and 30 % of the 7DHC present in the skin samples was turned into D_3, resulting in a total endogenous production of D_3 between 0·125 and 0·375 μg/cm^2 in rat skin and between 0·75 and 2·25 μg/cm^2 in pig skin[8,9]. Studies with human skin samples, expressing varying degrees of pigmentation and exposed to different UV light intensities, showed that the formation of preD_3 from 7DHC was non-linear and reached a plateau when 10–15 % of the 7DHC present in the skin was photoisomerised. This was irrespective of the degree of pigmentation, but the necessary exposure time increased as pigmentation darkened[10,11]. An *in vitro* method, utilising the exposure of solutions of pure 7DHC in ethanol to UV light

Abbreviations: 25(OH)D_3, 25-hydroxycholecalciferol; 7DHC, 7-dehydrocholesterol; D_3, vitamin D_3; preD_3, pre-cholecalciferol.

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and subsequent measurement of the amount of D_3 produced, also revealed a non-linear relationship between the UV light dose given and the resulting D_3 synthesis[6].

However, none of these *in vitro* methods allowed for prediction of the impact of UV light or sunlight irradiation on the 25(OH)D_3 status of living organisms. If a direct link between UV light or sunlight exposure and 25(OH)D_3 status in plasma *in vivo* should be established, it would involve completely restricted access to UV light sources and sunlight during the experimental period, a diet void of D_3 and a possibility for easy blood sampling. Such a study would be difficult to perform in wild herbivores. Furthermore, the relationship between sunlight exposure and the resulting 25(OH)D_3 status is of interest to both wild herbivores and domestic livestock reared outside; especially livestock in organic production, where the use of artificial feed additives, including synthetic vitamins, is restricted to fulfil the principles of sustainable organic production[12].

Recently we showed that cows, in contrast to common notion, synthesise D_3 in their entire skin surface when exposed to summer sunlight[13]. Furthermore, commercial dairy cows are easy to control with respect to D_3 supply in contrast to wild herbivores, because their daily routines vary extremely little and they do not obtain any D_3 from sources other than the sun at pasture or synthetic feed additives in the barn. Hence, the present study was carried out in black and white Danish Holstein cows.

The aim of the present experiment was to investigate the necessary time-interval that large herbivores, exemplified by dairy cows, must have during access to pasture in midsummer at 56°N in order to maintain a constant 25(OH)D_3 status in plasma, and to investigate the suggested non-linear relationship between sunlight exposure time and 25(OH)D_3 status in plasma *in vivo*.

Materials and methods

Animals and management

The study complied with the Danish Ministry of Justice Law no. 1306 (23 November 2007) concerning experiments with animals and care of experimental animals and was under the supervision of the Danish Animal Experiments Inspectorate. A total of twenty dairy cows of black and white Danish Holstein breed in first lactation with an average yield of 27·5 (SEM 0·8) kg energy-corrected milk per d[14] were used. The animals were divided into five treatment groups according to their daily energy-corrected milk yield and dominant coat colour (black or white), and let out for pasture daily during different time intervals at daytime. In the remaining time, the animals were housed in tie stalls without access to sunlight. In the barn, the feed consisted of an *ad libitum* maize- and grass clover silage-based total mixed ration without added D_3. The total mixed ration was given once daily at 09.00 hours and milking was carried out twice daily at 06.00 and 17.00 hours. The study was conducted from 3 June to 30 June 2010 at the Department of Animal Science, Aarhus University in Tjele, Denmark at 9·3°E/56·3°N. Median, minimum and maximum values of global radiation in the geographical area during the study period are shown in Fig. 1. The data were obtained from the meteorological database at Aarhus University.

Treatments

Treatments consisted of five different durations of daily access to pasture: 15, 30, 75, 150 and 300 min. Animals with 300 min daily access to pasture were let out at 10.45 hours followed by the other treatment groups in reverse access time order, so that all animals were at pasture at sun zenith at 13.23 hours. Then,

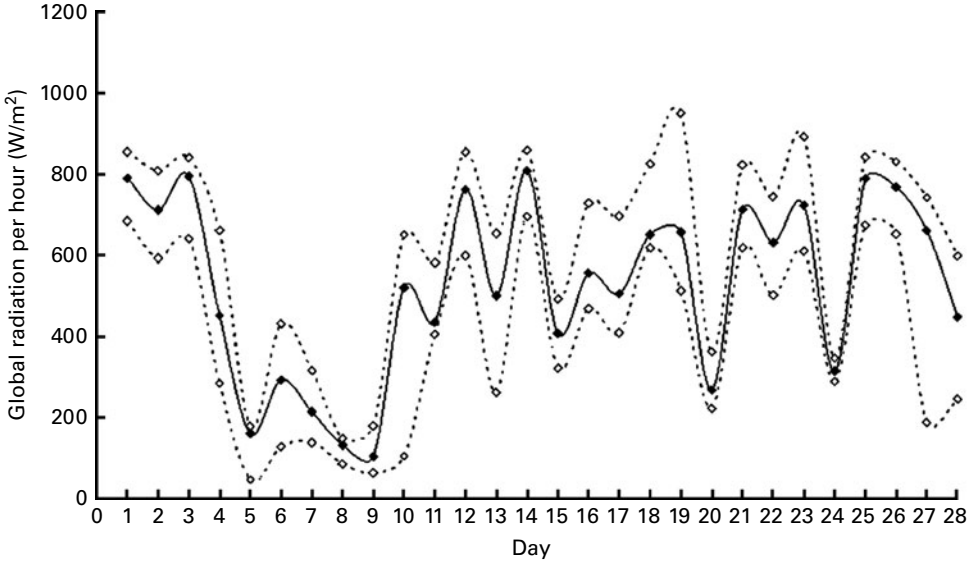

Fig. 1. Global radiation per hour (median, minimum and maximum values) during the time dairy cows spent at pasture each day of the study period in June 2010 at 56°N (data from the meteorological database at Aarhus University).

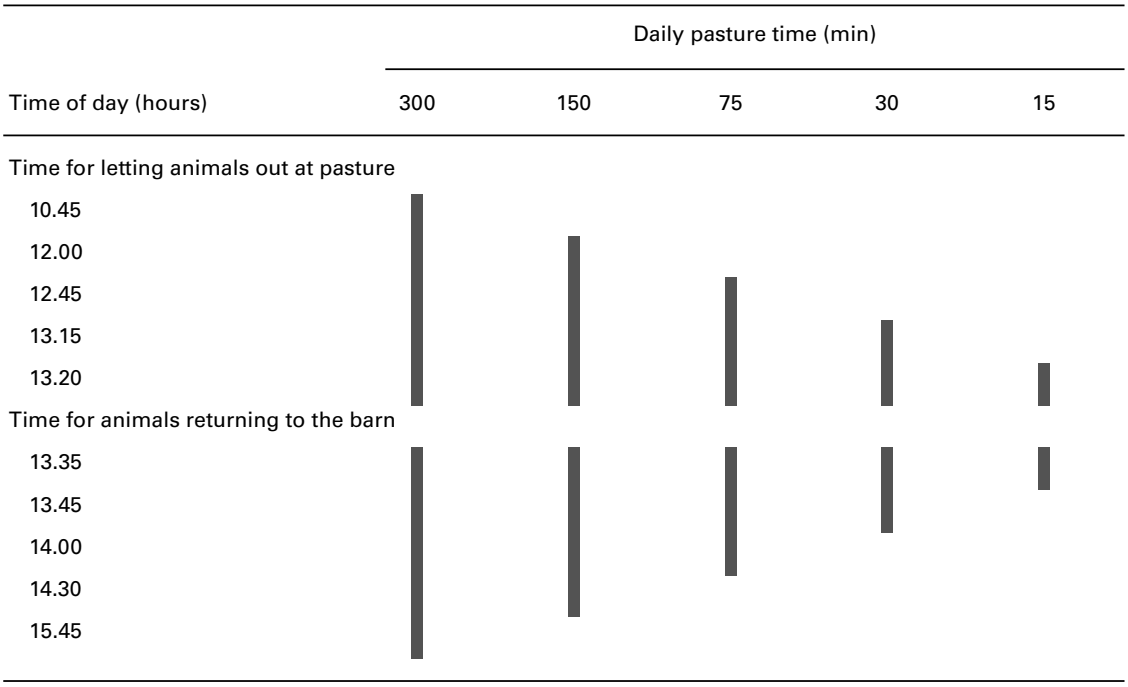

Fig. 2. Study design. ■, Animals at pasture.

the animals returned to the barn in the opposite order in which they were let out for pasture, with animals in the 300 min treatment group returning to the barn last at 15.45 hours (Fig. 2). From the onset of the study, synthetic D_3 was omitted from the total mixed feed ration used in the barn, rendering the animals with endogenous D_3 from the sunlight as their only source of D_3.

Blood samples

Blood was collected from the tail vein in Na-heparin-coated Vacuette® tubes (Greiner Bio-One GmbH, Kremsmünster, Austria). Samples were collected on days 0, 1, 3, 5, 7, 9, 12, 14, 16, 19, 21, 23, 26 and 28 of the study between 08.00 and 09.00 hours, except for the day-1 sample, which was taken

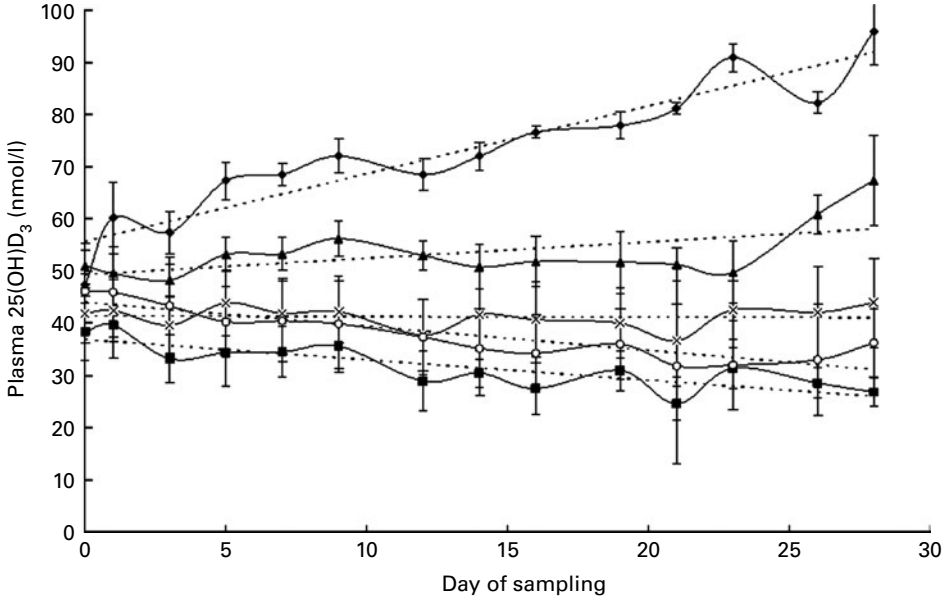

Fig. 3. Plasma concentrations of 25-hydroxycholecalciferol (25(OH)D_3) in dairy cows after daily access to pasture for different time durations in June 2010 at 56°N in Denmark. Values are means, with standard errors represented by vetical bars. ·····, Linear trend line; —◆—, 300 min; —▲—, 150 min; —×—, 75 min; —■—, 30 min; —●—, 15 min.

Table 1. Linear equations and R^2 values of 25-hydroxycholecalciferol (25(OH)D_3) plasma concentration curves (see Fig. 3) from dairy cows after daily access to pasture for different time durations in June 2010 at 56°N in Denmark

Daily pasture time (min)	Linear equation	R^2
300	$y = 1{\cdot}30x + 55{\cdot}6$	0·88
150	$y = 0{\cdot}31x + 49{\cdot}2$	0·33
75	$y = -0{\cdot}01x + 41{\cdot}3$	0·002
30	$y = -0{\cdot}39x + 36{\cdot}8$	0·68
15	$y = -0{\cdot}46x + 44{\cdot}0$	0·80

x, Daily pasture time (min); *y*, plasma concentration of 25(OH)D_3 (nmol/l).

at 16.00 hours after the animals returned from pasture on the first day of the study. Samples were centrifuged in a Thermo Scientific SL40 centrifuge (Thermo Scientific, Asheville, NC, USA) for 10 min at 1500 ***g*** and plasma was transferred to sample tubes (Sarstedt Group, Nümbrecht, Deutschland) and stored at −18°C until analysis.

Chemical analysis

In the laboratories at the Department of Animal Science, Aarhus University in Tjele, Denmark, the plasma samples were analysed for content of 25(OH)D_3, as described by Hymøller & Jensen[15].

Statistical analysis

ANOVA was performed in the MIXED models procedure of SAS® (SAS Institute, Inc., Cary, NC, USA) using the model: $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + C_{ijk} + \varepsilon_{ijk}$, where Y_{ijk} is the plasma concentration of 25(OH)D_3, μ is the overall mean, α_i is the fixed effect of i minutes daily access to pasture (15, 30, 75, 150, 300), β_j is the fixed effect of sampling day j (0, 1, 3, 5, 7, 9, 12, 14, 16, 19, 21, 23, 26, 28), $(\alpha\beta)_{ij}$ is the effect of the interaction between i minutes daily access to pasture and sampling day j, C_k is the random effect of animal k and ε_{ijk} is the random residual error. To account for the covariance of repeated measures during consecutive days within animals, the covariance structure was modelled using the repeated statement of the MIXED procedure of SAS®[16]. The best model fit was obtained from the autoregressive first-order covariance structure (AR(1)). To test for fit of a second-degree polynomial, a quadric term of α_i^2 was added to the model; however, as the effects of the quadric term were non-significant and the model fit statistics worsened, a linear model was maintained. Because the animals were grouped according to their dominant coat colour, the effects of coat colour on the repeated measures of plasma 25(OH)D_3 were tested by adding the fixed effect of coat colour and the interaction between coat colour and minutes of daily access to pasture to the model. Random effects were assumed normally distributed with mean value zero and constant variance C_{ijk} approximately $N(0, \sigma_c^2)$ and ε_{ijk} approximately σ^2. Results are presented as means with their standard errors and the differences considered statistically significant if $P \leq 0{\cdot}05$.

Results

The average concentration of 25(OH)D_3 in plasma at the beginning of the study across all treatment groups was 44·9 (SEM 2·4) nmol/l. At the end of the study at day 28, the 25(OH)D_3 concentrations were: 15 min: 36·2 (SEM 6·4) nmol/l, 30 min: 26·7 (SEM 2·8) nmol/l, 75 min: 43·9 (SEM 8·5) nmol/l, 150 min: 67·4 (SEM 8·6) nmol/l and 300 min: 95·9 (SEM 6·4) nmol/l. There was a significant effect of duration of daily pasture access ($P \leq 0{\cdot}001$) and day of sampling ($P \leq 0{\cdot}001$), as well as a significant interaction between duration

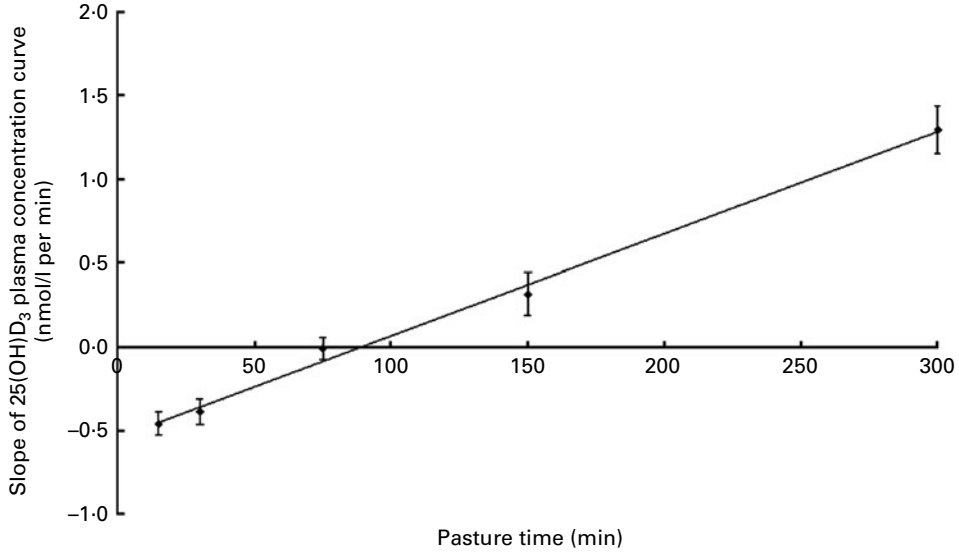

Fig. 4. Relationship between slope of linear 25-hydroxycholecalciferol (25(OH)D_3) concentration curves with standard errors (see Fig. 3) from dairy cows and daily time spent at pasture in June 2010 at 56°N in Denmark (intercept with *x*-axis 90·9 min). $y = 0{\cdot}006x - 0{\cdot}545$; R^2 0·995.

of daily pasture access and day of sampling ($P \leq 0{\cdot}001$). The developments in plasma concentrations of $25(OH)D_3$ within the five different treatments during the 28 d of study are shown in Fig. 3. Animals allowed 15, 30 or 75 min of daily access to pasture were unable to maintain their initial plasma status of $25(OH)D_3$ and showed a declining linear relationship between plasma $25(OH)D_3$ concentration and day of sampling in contrast to animals allowed 150 or 300 min of daily sunshine access, which showed an increasing relationship between plasma $25(OH)D_3$ and day of sampling (Fig. 3). All slopes of plasma concentration curves, except for the 75 min treatment group (slope $-0{\cdot}01$), were significantly different from zero in linear regression ($P \leq 0{\cdot}05$).

From the slopes of the average $25(OH)D_3$ concentration curves within treatments (Table 1), breakeven for maintaining the initial $25(OH)D_3$ status was determined to be approximately 90 min of pasture per d during mid-summer in Denmark at 56°N (Fig. 4). The dominant coat colour (black or white) of individual animals had no significant effect on the plasma concentrations of $25(OH)D_3$ within the different treatments.

Discussion

In cattle, the plasma status of $25(OH)D_3$ was shown by Hymøller & Jensen[13] to be closely related to the percentage of total skin area that was exposed to sunlight; and Barger-Lux & Heaney[17] showed that the size of the exposed skin area was more closely correlated to the plasma status of $25(OH)D_3$ than the duration of sunlight exposure in human subjects. In the present study, the plasma $25(OH)D_3$ status of black and white Holstein dairy cows was strongly linearly dependent on the time the animals spent at pasture on a daily basis during summer at 56°N in Denmark. This contradicts previous assumptions based on *in vitro* studies where the relationship between sunlight or UV light exposure time and the formation of D_3[6] and $preD_3$ from 7DHC was shown to be non-linear and to reach a plateau when a fraction of the 7DHC present in the skin was photoisomerised[10,11]. The plateau phenomenon encountered when studying D_3 synthesis during different durations of UV light exposure *in vitro*, with either skin samples or pure solutions of 7DHC, is probably caused by a photocatalysed degradation of the intermediate metabolite of D_3, $preD_3$, when exposed to excessive amounts of UV light, before it had time to be isomerised into D_3, catalysed by heat at body temperature[3,5,18].

The strong linear relationship between plasma $25(OH)D_3$ and pasture time at 56°N in the present study could be due to non-production of inactivated metabolites of D_3 in the skin. There could be different explanations supporting this: The altitude at 56°N could be so far north that the sunlight intensity was significantly reduced compared to lower latitudes[5]; the hair covering the bodies of the animals could have protected their skin from direct exposure to sunlight; or the blood flow through the skin of living organisms could have transported the produced D_3 away from the skin and into circulation in plasma, before it could be photodegraded by excessive sunlight exposure.

Even though it has been shown in both human subjects[10,11] and in fur- or hair-covered species like rats[19] and alpacas[20] that dark-coloured individuals respond slower to sunlight than light-coloured individuals, no effect of dominant coat colour was found in the present study with black- and white-coloured animals.

In conclusion, our study showed that plasma concentrations of $25(OH)D_3$ were linearly correlated to day of sampling within each treatment group allowed daily access to pasture for different time intervals. Animals allowed 15, 30 or 75 min of daily access to pasture were unable to maintain their initial plasma status of $25(OH)D_3$ and showed a declining relationship between plasma $25(OH)D_3$ concentration and day of sampling in contrast to animals allowed 150 or 300 min of daily sunshine access, which showed an increasing relationship between plasma $25(OH)D_3$ and day of sampling. Breakeven for maintaining the initial $25(OH)D_3$ status of 45 nmol/l in large, hair-covered herbivores was determined to be 90 min of access to pasture per d during summer at 56°N. The dominant coat colour of individual animals (black or white) had no effect on the plasma concentrations of $25(OH)D_3$.

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