Dehydroepiandrosterone secretion in dairy cattle is episodic and unaffected by ACTH stimulation

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Abstract

This paper describes the episodic release and response to adrenal stimulation of cortisol and dehydroepiandrosterone (DHEA) in cows. Observations made in samples taken every 10 min for 8 h (experiment 1) showed that plasma DHEA was significantly greater ($P < 0.001$) than DHEA-S, and release of these steroids was episodic and variable between animals ($P < 0.01$). No relationship was found between DHEA and cortisol. Significant ($P < 0.001$) DHEA-sulphate (DHEA-S) versus cortisol ($R = -0.264$) and DHEA-S versus DHEA ($R = 0.200$) correlations were found. DHEA and DHEA-S were not affected by a single ACTH challenge (experiment 2). In experiment 3, cortisol and DHEA secretions in response to prolonged ACTH administration (every 12 h for 6 days) were studied. On day 7, the episodic cortisol and DHEA release and response to the opioid antagonist naloxone were studied in blood samples taken every 10 min for 8 h. Animals were

injected with naloxone after 4 h. A significant increase ($P < 0.05$) in mean circulating DHEA and DHEA pulse amplitude was observed during frequent sampling following ACTH treatment. DHEA and DHEA-S plasma concentrations were not affected following luteal regression (experiment 4). The effect of milk secretion around parturition on DHEA secretion was studied in dry and continuously milked cows (experiment 5). Plasma DHEA was significantly lower ($P < 0.05$) in milked cows. In the cow, ACTH is not an important DHEA secretagogue. Adrenal contribution to plasma DHEA is scarce. Likely, the placenta is the most important source of DHEA, and the lactating mammary gland can affect circulating DHEA levels. Investigation about the DHEA biological role in cows should be focused around parturition.

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Introduction

Dehydroepiandrosterone and its sulphate (DHEA-S) are secreted mainly by the adrenals of primates and serve as intermediates in the synthesis of androgens and estrogens in peripheral tissues (Labrie *et al.* 2005). They are the most abundant steroids in the human circulation, and they have been associated with several biological actions beneficial for human health and to the physiological and pathological phenomena connected to the ageing process (Roberts 1999, Labrie *et al.* 2005). In primates and rodents, DHEA is also a neurosteroid and it is produced within the CNS and in peripheral nerves, where it acts as an allosteric modulator of γ -amino butyric acid type A (GABA-A) and sigma receptors, and it is associated with several behavioural circumstances and neurodegenerative diseases (Baulieu 1998, Zinder & Dar 1999, Maurice *et al.* 2001, Labrie 2005).

DHEA is implicated in the stress response (Zinder & Dar 1999) and the adrenal of the adult human responds to stress with increased cortisol and diminished DHEA/DHEA-S secretion (Parker 1999). In rats, DHEA administration antagonises the effects of repeated immobilisation stress (Hu *et al.* 2000).

DHEA and its metabolites play a role in the immune system activation, counteracting the immunosuppressive action of cortisol (Loria *et al.* 1996). DHEA and DHEA-S have anti-inflammatory effects probably mediated by their antioxidant properties, which prevent the activation of the pleiotropic transcription factor nuclear factor- κ B (NF- κ B; Iwasaki *et al.* 2004, Aragno *et al.* 2006). The antioxidant properties of DHEA have been demonstrated both *in vivo* (Pelissier *et al.* 2004) and *in vitro* (Bastianetto *et al.* 1999).

DHEA is also involved in lipid metabolism, showing protective effects against visceral fat accumulation and muscle insulin resistance development (Hansen *et al.* 1997) and, likely, it exerts its fat-reducing effects by increasing peroxisome proliferator-activated receptor- α (PPAR α) gene expression and consequently inducing the expression of enzymes involved in fatty acid catabolism (Kochan & Karbowska 2004).

Some findings raised our interest in the possible biological role of DHEA in the female bovine. First of all, the bovine mammary gland can convert DHEA into androstene-3 β ,17 β -diol (AED; Belvedere *et al.* 1996). Then, plasma DHEA and AED concentrations are higher in late pregnancy and drop

suddenly after parturition (Gabai *et al.* 2004). Finally, it was recently reported that cows with inflammatory foot lesions showed a 23% decrease in serum DHEA concentrations (Almeida *et al.* 2007), suggesting that DHEA is implicated in the inflammation process also in the bovine.

In an early study (Feher *et al.* 1977), it was observed that DHEA was present in the plasma of most mammals at nanomolar levels, and the concentrations of this steroid in animal plasma were similar or higher than those in human subjects. On the contrary, DHEA-S was present in human plasma at micromolar levels, and in plasma of rat, dog and pig at nanomolar levels, while it was undetectable in plasma of ruminants, guinea pig and rabbit.

In humans, DHEA and DHEA-S display a diurnal rhythm in parallel to that of cortisol (Lejeune-Lenain *et al.* 1987), and it was hypothesised that social activity and living habits may play a role in the synchronisation of diurnal DHEA/DHEA-S rhythms (Zhao *et al.* 2003). In the human, adrenal DHEA secretion is episodic and it is synchronous with cortisol release, while DHEA-S seems to be less synchronous with both DHEA and cortisol (Rosenfeld *et al.* 1975). In the human male, it was observed that the pulsatility of the corticotrophic axis was transmitted to the secretory pattern of DHEA, and a partial synchronisation between testicular and adrenal pulsatile secretion was hypothesised (Lejeune-Lenain *et al.* 1987).

In humans, DHEA secretion is responsive to ACTH stimulation (Parker 1999, Arvat *et al.* 2000). However, in one study performed on healthy women (Azziz *et al.* 2001), it was observed that DHEA levels in response to ACTH stimulation were extremely variable among subjects. It is evident that the control of cortisol and DHEA secretion frequently diverges, and substances of pituitary origin other than ACTH were hypothesised to control adrenal androgen secretion (Odell & Parker 1985).

To our knowledge, little information about DHEA and DHEA-S secretion in the dairy cow is available in the literature and, in particular, the adrenal contribution to DHEA secretion has been poorly investigated. The aim of this work was to extend the knowledge about the secretion of DHEA and DHEA-S in the dairy cow and, in particular, we aimed to study a) the pattern of DHEA and DHEA-S secretion, b) their response to exogenous ACTH administration and c) the effects of phase of the oestrus cycle (luteal versus follicular phases) and lactation on DHEA secretion.

Materials and Methods

Animals and procedures

Animals were trained to frequent handling for at least 1 month before each experiment, and animal health conditions were assessed by clinical examination during the whole experimental periods. All procedures were carried out in respect of the Italian legislation on animal care (DL n.116, 27/1/1992).

Experiment 1: pattern of secretion

This experiment was designed to study the episodic release of cortisol, DHEA and DHEA-S in the dairy cow. Six lactating non-pregnant Italian Friesian cows in their third month of lactation were used. Animals were housed in tie stalls, with almost constant climate conditions, daylight interval and feed-diet characteristics. Cows were individually fed *ad libitum*; forages (maize silage, alfalfa and grass hay) were offered in two meals (0730 and 1930 h), and concentrate in eight meals by auto feeder. The cows were milked twice a day at 0500 and 1700 h. Indwelling jugular catheters were inserted under local anaesthesia the day before the experiment, and blood samples were taken every 10 min for 8 h, beginning at 1000 h. Blood samples were kept refrigerated and plasma was obtained by centrifugation (1500 g, 4 °C, 15 min) within 30 min.

Experiment 2: response to single ACTH challenge

The experiment was carried out on four non-lactating non-pregnant multiparous Friesian cows to study the short-term response of cortisol, DHEA and DHEA-S to ACTH challenge. Animals were housed in individual boxes under natural light condition. Cows were fed once a day a total mixed ration (TMR) and had free access to water. The cows were randomly assigned to treatments according to a crossover experimental design, consisting of two experimental periods with a 40-day resting period in between. Treatments consisted of the i.m. administration of either saline (control; CTR) or an ACTH analogue (tetracosactrin acetate, Synacthen depot, Novartis Pharma; 0.5 mg; ACTH), and the treatment administration was referred as time 0. The plasma concentrations of steroids were measured in plasma samples obtained from the jugular vein before 8 h and at 0, 1, 2, 4, 9 and 12 h from treatment administration.

Experiment 3: response to long-term ACTH administration

The experiment was carried out on five non-lactating non-pregnant multiparous Friesian cows to study the response of cortisol and DHEA to long-term adrenal stimulation.

Animals were housed in tied stalls under natural light condition, were fed a standard diet consisting of hay and concentrate, and had free access to water. The experimental protocol consisted of a control phase (CTR) followed by a treatment phase (ACTH) with a resting period of at least 30 days in between. Animals received i.m. injections of either saline (CTR) or ACTH analogue (Tetracosactrin acetate, Synacthen depot, Novartis Pharma; 0.7 mg/head/administration; ACTH) every 12 h per 6 days. The beginning of the treatment was indicated as day 0. Blood samples were collected from the jugular vein in heparinised tubes and they were kept refrigerated until plasma was obtained by centrifugation (1500 g, 4 °C, 15 min) within 30 min. Plasma was divided in aliquots and stored at -20 °C until required for analysis. Samples were taken with the same time intervals

during the control and treatment phases to measure the concentrations of circulating cortisol and DHEA the day before and on days 0, 1, 4, 5 and 11.

On day 7, the pulsatile cortisol and DHEA release and response to the opioid antagonist naloxone were studied in blood samples taken every 10 min for 8 h. Indwelling jugular catheters were inserted under local anaesthesia the day before (day 6), and frequent sampling begun at 1000 h. After 4 h, the animals received an injection of naloxone hydrochloride (1 mg/kg body weight i.v.; Sigma) dissolved in saline.

Experiment 4: steroid variation around luteal regression

This experiment was performed to study cortisol, DHEA and DHEA-S plasma concentrations around the period of the induced luteal regression, in order to study the ovarian influences on the plasma levels of these steroids. The experiment was carried out on four non-lactating non-pregnant multiparous Friesian cows housed in individual boxes under natural light condition. Cows were fed once a day a TMR and had free access to water. The cows were treated with two injections of prostaglandin (PG; 2 mg cloprostenol, Estrumate, Schering-Plough, Uxbridge, UK) administered 11 days apart, and the second PG administration was referred as time 0. Plasma concentrations of cortisol, DHEA and DHEA-S were measured in samples taken from the jugular vein every 12 h, from -60 to 0 h, and at 4, 12, 24, 36, 48, 60, 110, 120 and 140 h.

Experiment 5: response to continuous lactation

The last experiment was carried out in Holstein cows subjected to a 55-day dry period (CTRL, $N=5$) and continuously milked cows (MILK, $N=6$) to study the effect of mammary gland activity on the secretion of cortisol and DHEA. The animals were assigned to each experimental group to have homogeneous average milk yield, expressed as mature equivalent cow (CTRL = 105 ± 12 ton; MILK = 109 ± 14 ton). The groups were placed in two distinct pens. The cows in the MILK group were milked until parturition. The cows of the CTRL group were dried-off 55 days before parturition. All cows had free access to water and were fed once daily. Blood samples were collected before parturition (at days -60, -55, -40, -30 and -7) and after parturition (at days 1, 7, 15, 30, 60 and 90).

Hormone assays

Plasma cortisol (C) was measured using a solid phase microtitre RIA after diethyl ether extraction (Gabai *et al.* 2006). All samples were assayed in duplicate. The intra- and inter-assay coefficients of variation (CV) were 3.1 and 2.8% respectively.

Plasma DHEA was measured by the RIA described elsewhere (Gabai *et al.* 2004). The intra- and inter-assay CV were 8.7 and 10.0% respectively.

Plasma DHEA-S concentrations were analysed by a solid-phase microtitre direct RIA. Briefly, a 96-well microtitre plate Optiplate, Perkin-Elmer Life Science) was coated with anti-rabbit γ -globulin serum raised in a goat, incubating overnight the antiserum diluted 1:1000 in 0.15 M sodium acetate buffer, pH 9, at 4 °C. The plate was then washed twice with PBS, 0.1% BSA, pH 7.4 (RIA buffer) and incubated overnight at 4 °C with 200 μ l of the anti-DHEA-S serum diluted 1:8000. The antiserum (Spi Bio, Montigny, France) was raised in the rabbit against DHEA-S-7-carboxymethyl-oxime-BSA and showed the following cross reactions: DHEA-S 100%, Δ 4-androstenedione 0.2%, DHEA, testosterone and androsterone 0.01%. The plate was carefully washed with RIA buffer, and standards (1.56–400 pg/well/50 μ l), quality control, unknown samples (20 μ l) and tracer (1,2,6,7- 3 H-DHEA-S, Perkin-Elmer Life Sciences, 30 pg/well/10 μ l, specific activity: 2927 GBq/mmol) were added. The final volume of the reaction mixture was adjusted to 200 μ l with RIA buffer. The plate was incubated overnight at 4 °C, the incubation mixture was decanted and wells washed with RIA buffer, added with 200 μ l scintillation cocktail (Microscint 20, Perkin-Elmer Life Sciences) and counted on the β -counter (Top-Count, Perkin-Elmer Life Sciences). The DHEA-S RIA was validated by performing parallelism and recovery tests. Parallelism data were expressed as the regression curve between observed hormone concentrations and the reciprocal of the dilution factors of serially diluted plasma samples ($(H_{\text{obs}}) = a \times 1/df + b$). The assay showed a good degree of parallelism, as demonstrated by the values of the intercept (b) that was not significantly different from 0. Recovery was expressed as the regression curve between observed and expected hormone concentrations ($(H_{\text{obs}}) = a(H_{\text{exp}}) + b$) measured in plasma samples spiked with known amount of DHEA-S. The angular coefficient (a) was not significantly different from 1 and the intercept (b) was not significantly different from 0 indicating a good correspondence between expected and observed hormone concentrations. The sensitivity of the assay expressed as 90% of hormone bound was 3.1 pg/well. The intra- and inter-assay CV were 6.7 and 8.3% respectively.

Data analysis

The pulsatile cortisol and DHEA release was analysed by the computer program 'Pulsar' based on the algorithm developed by Merriam & Watcher (1982), which calculated the amplitude and frequency of hormone pulses, baseline concentrations and inter-peak intervals. Cut-off criteria (G(1) to G(5)) were set to keep the probability of getting a false positive equal to or less than 5%.

Data are expressed as mean $\pm$ standard error. Hormone data underwent ANOVA by the general linear model procedure of SPSS. When appropriate, differences between means were analysed by Student *t*-test or Duncan test. In experiments 2, 3 and 5, independent variables were the treatment (*T*), sampling

time (ST), animal (A) within *T* and the *T*×*ST* interaction. In experiment 3, data measured before and after naloxone administration were recorded separately and were analysed by ANOVA. Terms of the models were *T*, the period (*P*, before naloxone administration versus after naloxone administration), *A* within *T* and the *T*×*P* interaction. In experiment 4, terms of the model were *ST* and *A*. Correlation between cortisol, DHEA and DHEA-S plasma concentrations were expressed as Pearson correlation coefficients (SPSS, Chicago, IL, USA).

Results

Experiment 1: pattern of secretion

The parameters describing the episodic release of cortisol, DHEA and DHEA-S over a period of 8 h (between 1000 and 1800 h) in six non-pregnant dairy cows in their third month of lactation are reported in Table 1. The profiles of plasma DHEA and DHEA-S concentrations in the six cows are reported in Fig. 1. On average, plasma DHEA concentrations were significantly higher than plasma DHEA-S concentrations ($P<0.001$). The three steroids showed different patterns of episodic release. Plasma DHEA-S showed a weak but significant degree of correlation with both plasma cortisol ($R=-0.264$, $P<0.001$) and DHEA ($R=0.200$, $P<0.001$), while plasma cortisol and DHEA did not correlate each other.

Experiment 2: response to single ACTH challenge

The administration of the ACTH agonist induced a significant increase ($P<0.01$) of plasma cortisol within 1 h after injection. Circulating cortisol returned to basal values within 10 h after the drug administration (Fig. 2). On the contrary, both circulating DHEA and DHEA-S were not affected by the ACTH agonist (Fig. 2) and showed a significant cow effect ($P<0.01$). A weak but significant degree of correlation ($R=0.202$, $P<0.01$) was found between DHEA and DHEA-S.

Experiment 3: response to long-term ACTH administration

During the ACTH phase, plasma cortisol was significantly higher ($P<0.05$; $P<0.01$) from day 1 to day 7, while plasma

DHEA was not affected by treatment (Fig. 3). No correlation was found between cortisol and DHEA.

Parameters describing cortisol release during frequent sampling (day 7) are reported in Table 2. Mean plasma cortisol was still significantly higher after the end of the ACTH phase ($P<0.05$). Cortisol peak amplitude, peak frequency and inter-peak interval were affected by treatment ($P<0.05$). During the CTR phase, a cortisol pulse was observed within 30 min from naloxone administration in all animals. No apparent relationship between naloxone administration and cortisol pulse appearance was observed after the ACTH phase.

During frequent sampling, the mean concentrations and peak amplitude of plasma DHEA were significantly higher ($P<0.05$) in one cow, which was removed from the statistical analysis. The parameters describing DHEA release during frequent sampling (day 7) are reported in Table 3. Mean DHEA plasma levels were significantly higher after the ACTH phase ($P<0.05$), but no response to naloxone administration was observed after either CTR or ACTH phases.

During frequent sampling, plasma cortisol was not correlated to plasma DHEA in either the CTR or ACTH phases.

Experiment 4: steroid variation around luteal regression

Plasma concentrations of DHEA and DHEA-S were significantly different between individual cows ($P<0.01$). Circulating cortisol, DHEA and DHEA-S were not affected by the PG administration and no effect of the sampling time was observed (Fig. 4). A weak degree of correlation was found between DHEA and DHEA-S ($R=0.216$, $P<0.05$).

Experiment 5: response to continuous lactation

During the prepartum period, plasma DHEA concentrations significantly ($P<0.05$) increased in both groups. In the CTRL group, plasma DHEA was significantly ($P<0.05$) higher between D-30 and D1 in comparison to D-60. In the MILK group, plasma DHEA was significantly ($P<0.05$) higher on D-7 than on D-60. Then, plasma DHEA decreased to concentrations lower than 1 nM in both groups within 1 week after calving ($P<0.01$). Plasma DHEA concentrations were significantly lower in continuously milked cows at D-7 ($P<0.05$). As expected, plasma cortisol showed a significant

Table 1 Parameters describing the episodic release of cortisol, dehydroepiandrosterone (DHEA) and DHEA-sulphate (DHEA-S) over a period of 8 h (between 1000 and 1800 h) in non-pregnant dairy cows ($n=6$) in their third month of lactation

	Cortisol	DHEA	DHEA-S
Mean concentration (nM)	11.8±1.5	2.3±0.1	0.8±0.2
Peak amplitude (nM)	18.5±1.6	1.2±0.1	0.4±0.1
Peak duration (min)	30±2	24±2	22±2
Inter-peak interval (min)	104±9	76±5	77±6
Peak frequency (peak <i>n</i> /8 h)	4.3±0.2	6.5±0.3	5.7±0.4

Figure 1 Plasma DHEA (●) and DHEA-S (○) concentrations in six non-pregnant dairy cows during the third month of lactation measured over a period of 8 h (1000–1800 h) at 10-min intervals.

increase around parturition ($P < 0.01$), but it was not affected by continuous milking (Fig. 5).

Discussion

Adrenal androgens play several important roles in primate mammals as they are implicated in the control of several biological functions (see Loria *et al.* 1996, Nestler 1996, Roberts 1999, Labrie *et al.* 2005). Also in laboratory animals, if administered at pharmacological doses, DHEA exerts its biological activities (Nestler 1996). Hence, this steroid can maintain a biological function also in non-primate mammals, despite the lower concentration observed in the circulation of those species in comparison with primates (Feher *et al.* 1977). However, little information about the physiological role of these steroids in non-primate mammals is available.

In the female bovine, circulating DHEA may serve as a source of precursor for androgen and oestrogen synthesis. For instance, the mammary gland can use DHEA to make androstene- $3\beta,17\beta$ -diol (AED; Belvedere *et al.* 1996), a metabolite with immunoenhancing activity (Loria *et al.* 1996). In addition, it was recently reported that cows with inflammatory foot lesions showed a 23% decrease in serum DHEA concentrations (Almeida *et al.* 2007), suggesting that DHEA is implicated in the inflammation process also

in the bovine. Those observations, however, are not sufficient to hypothesise a biological role for DHEA in that species, and a better characterisation of the DHEA secretion is necessary.

In cows, circulating DHEA-S is significantly lower than DHEA, and DHEA release is very different among individual cows. Our data are consistent with previous observations in the bovine, where circulating DHEA-S was lower than in humans, and DHEA was more concentrated than DHEA-S in plasma (Feher *et al.* 1977). For this reason, in cows as in most non-primate mammals DHEA could be considered an indicator of the P450c17 enzyme activity and the most important circulating precursor of ectopic androgen and oestrogen synthesis, while the DHEA-S contribution as an androgen reservoir is rather limited.

The relationship between the secretion of DHEA and cortisol has not been fully understood. Our observations in cows showed that the secretion of cortisol, DHEA and DHEA-S is episodic. Cortisol and DHEA pulse frequency observed in cows was comparable with that observed in humans (Lejeune-Lenain *et al.* 1987), where DHEA and cortisol episodic secretions showed a strong overall synchronisation (Rosenfeld *et al.* 1975, Lejeune-Lenain *et al.* 1987). In particular, both steroids showed a bi-modal circadian pattern with a major acrophase in early morning and a late evening nadir, and a high degree of correlation and

Figure 2 Cortisol, DHEA and DHEA-S plasma concentrations measured in four non-pregnant, non-lactating dairy cows treated with a single injection i.m. of saline (CTR, ○) or ACTH agonist (ACTH, ●). The asterisks indicate statistically significant differences between CTR and ACTH (** $P < 0.01$; Student *t*-test).

concomitance of episodic pulses (Lejeune-Lenain *et al.* 1987). In this study, frequent sampling was performed for 8 h from the morning to early afternoon, and this was not suitable to detect any circadian pattern of steroid release. During the observation period, however, no relationship between cortisol and DHEA secretion was found in the cow, suggesting that cortisol and DHEA release are desynchronised. We are not aware of any studies on the pattern of DHEA/DHEA-S release in non-primate mammals and, in this respect, our results bring an element of novelty.

Figure 3 Plasma cortisol and DHEA concentrations measured in five non-pregnant, non-lactating cows treated with either saline (CTR, ○) or an ACTH agonist (ACTH, ●) administered every 12 h for 6 days. The black bar indicates the treatment administration. The asterisks indicate statistically significant differences between CTR and ACTH (* $P < 0.05$; ** $P < 0.01$, Student *t*-test).

Although some *in vitro* studies showed that the bovine adrenals do secrete DHEA and respond to ACTH (Higuchi *et al.* 1985, Judd *et al.* 2000), our data strongly suggest that ACTH has a negligible effect on DHEA and DHEA-S release in the bovine *in vivo*.

In humans, DHEA secretion is responsive to ACTH stimulation (Parker 1999), although some observations are still controversial. In a dose-response study (Arvat *et al.* 2000), it was observed that plasma DHEA increased in response to a minimum dose of 0.01 µg ACTH and the response was similar in male and female subjects. Indeed, those authors concluded that DHEA was more sensitive than cortisol to ACTH stimulation.

However, in numerous situations described in humans, DHEA secretion is modulated separately from ACTH and diverges from that of cortisol (Odell & Parker 1985, Azziz *et al.* 2001). For example, the adrenal of the adult human responds to stress with increased cortisol and diminished DHEA/DHEA-S secretion (Parker 1999). In another study performed in healthy reproductive-aged women (Azziz *et al.* 2001), it was observed that both basal and ACTH-stimulated DHEA release had a very high inter-subject variability. Those authors suggested that ACTH is only partially responsible for adrenal androgen secretion and factors other than ACTH are implicated in DHEA biosynthesis. A similar observation was reported in an early study (Odell & Parker 1985) performed in castrated dogs treated with either synthetic ACTH or bovine pituitary extract. In that experiment, the bovine pituitary extract contained a substance that was more potent than ACTH in stimulating DHEA release in the castrated dogs (Odell &

Table 2 Parameters describing the episodic release of cortisol over a period of 8 h (between 1000 and 1800 h) in non-pregnant dairy cows in their third month of lactation treated with either saline (CTR) or an adrenocorticotrophic hormone (ACTH) agonist every 12 h for 6 days. After 4 h, the cows received an injection of naloxone hydrochloride

	CTR		ACTH	
	Before naloxone	After naloxone	Before naloxone	After naloxone
Mean concentration (nM)	7.7 ± 0.6 ^a	8.7 ± 0.8 ^a	59.5 ± 2.4 ^b	52.5 ± 1.3 ^b
Peak amplitude (nM)	11.8 ± 2.6 ^a	13.1 ± 3.5 ^a	33.7 ± 5.1 ^b	25.4 ± 2.4 ^b
Peak duration (min)	29 ± 2	27 ± 8	24 ± 3	26 ± 3
Inter-peak interval (min)	77 ± 24 ^b	84 ± 10 ^b	59 ± 7 ^a	51 ± 6 ^a
Peak frequency (peak n/4 h)	2.8 ± 0.6 ^a	2.2 ± 0.2 ^a	4.2 ± 0.2 ^b	4.2 ± 0.5 ^b

Different superscripts in the same row indicate significantly different means within treatment (^{a, b} $P < 0.05$; Duncan test).

Parker 1985). Nevertheless, subsequent reports failed to confirm the existence of such a substance (Nestler 1996).

The low ACTH effect on DHEA release in the cow was indirectly confirmed by naloxone administration. In the absence of adrenal stimulation by exogenous ACTH, the injection of naloxone induced a rise of circulating cortisol within 30 min, suggesting that the opioid antagonist dose was adequate to induce the release of CRH/ACTH. On the contrary, no DHEA response was observed following naloxone administration. Likely, naloxone acted centrally to overcome the opioid blockade of the CRH/ACTH release that induced a cortisol pulse, but it was not sufficient to stimulate DHEA release.

This is in agreement with what has been observed in other species. Human and animal studies have established that endogenous opioids can inhibit the hypothalamus–pituitary–adrenal (HPA) axis, and naloxone administration can stimulate ACTH and glucocorticoids secretion (Jessop 1999). On the other hand, acute opioid blockade failed to induce a DHEA-S response in humans, although it was associated with cortisol release (Ceballos *et al.* 2007).

In our work, when the cows were treated with exogenous ACTH, no cortisol or DHEA response to naloxone was detected. In this case, the ACTH treatment resulted in a massive cortisol release that, likely, masked the effect of naloxone. In addition, it is probable that exogenous ACTH impaired the feedback mechanism controlling CRH/ACTH release, thus impeding any effect of naloxone. However, long-term exogenous ACTH administration resulted in an elevation of mean plasma concentration and pulse amplitude of DHEA, which were detectable only by frequent sampling.

Taken together, our data indicated that ACTH is not an important secretagogue for DHEA in the cow, and the adrenal contribution to plasma DHEA is scarce. However, the concentrations and secretory pattern of this steroid could be very different between individual animals, suggesting that some individuals may be more sensitive to ACTH. Alternatively, DHEA secretion could be more intense in particular physiological conditions not evidenced in this work, and it could be under the control of other mechanisms involving factors different from ACTH.

In humans, insulin can reduce circulating DHEA and DHEA-S (see Nestler 1996), but we are not aware about the relationship between insulin and DHEA in other species. In the periparturient cow, we found a small but significant negative correlation between insulin and DHEA ($R = -0.225$, $P < 0.01$, unpublished data) that seems to agree with the observations made in humans. Nevertheless, endocrine modifications in the periparturient cow are so complex that it is unworthy to consider this observation physiologically relevant.

Other factors can affect adrenal steroidogenesis. For example, basal and ACTH-stimulated DHEA secretion from dispersed bovine adrenal cells can be enhanced by prolactin (Higuchi *et al.* 1985), and other proopiomelanocortin (POMC)-derived peptides, such as β -endorphin and joining peptide, can stimulate DHEA release from human adrenal cells (Clarke *et al.* 1996).

Also cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor α (TNF α), act as intra-adrenal modulators of adrenal steroid secretions (Nestler 1996, Judd *et al.* 2000, Bornstein *et al.* 2004). IL-6 and TNF α are expressed in the

Table 3 Parameters describing the episodic release of dehydroepiandrosterone (DHEA) over a period of 8 h (between 1000 and 1800 h) in non-pregnant dairy cows in their third month of lactation treated with either saline (CTR) or an adrenocorticotrophic hormone (ACTH) agonist every 12 h for 6 days. After 4 h, the cows received an injection of naloxone hydrochloride

	CTR		ACTH	
	Before naloxone	After naloxone	Before naloxone	After naloxone
Mean concentration (nM)	1.7 ± 0.1 ^a	1.7 ± 0.1 ^a	2.6 ± 0.1 ^b	2.4 ± 0.1 ^b
Peak amplitude (nM)	1.2 ± 0.2 ^a	1.1 ± 0.1 ^a	1.5 ± 0.3 ^b	1.6 ± 0.1 ^b
Peak duration (min)	22 ± 3	16 ± 2	22 ± 3	18 ± 3
Inter-peak interval (min)	73 ± 13	69 ± 10	73 ± 14	71 ± 7
Peak frequency (peak n/4 h)	3.0 ± 0.4	3.0 ± 0.1	2.2 ± 0.4	2.5 ± 0.3

Different superscripts in the same row indicate significantly different means within treatment (^{a, b} $P < 0.05$; Duncan test).

Figure 4 Cortisol ($\square$), DHEA ($\bullet$) and DHEA-S ($\circ$) plasma concentrations measured in four non-pregnant, non-lactating dairy cows around the time of induced luteal regression. Time 0 indicates the administration of the PGF $_{2\alpha}$ analogue. Pooled standard errors were cortisol=0.64 nM, DHEA=0.11 nM and DHEA-S=0.05 nM.

adrenal cortex of human (Nestler 1996, Judd *et al.* 2000, Bornstein *et al.* 2004), rat and bovine (Judd *et al.* 2000), but they show opposite actions in the human and the bovine. IL-6 stimulates cortisol secretion from both human and bovine adrenal cells, and it has a stimulatory action on DHEA secretions in human adrenal cells. On the contrary, IL-6 exposure inhibits basal and ACTH-stimulated DHEA secretion from bovine adrenal cells, which is also inhibited by TNF α (Judd *et al.* 2000).

Figure 5 Plasma cortisol and DHEA concentrations measured in cows with a 55-day dry period (CTRL, $n=5$, $\bullet$) and continuously milked cows (MILK, $n=6$, $\square$). The asterisk indicates a statistically significant difference between CTRL and MILK ($*P<0.05$, Student t -test).

In the non-pregnant cow, DHEA and DHEA-S presumably originate from the adrenals (Higuchi *et al.* 1985, Judd *et al.* 2000) and the gonads (Wise *et al.* 1982), although the relative contribution of these tissues is difficult to extrapolate. However, our results suggest that the ovaries do not secrete an amount of DHEA capable of significantly affecting its plasma concentrations at the time of induced ovulation, possibly because at that time the steroid is used for oestrogen synthesis (Wise *et al.* 1982).

Likely, the most important source of DHEA in the cow is the placenta, which utilises mainly the $\Delta 5$ steroidogenic pathway to produce oestrogen (Geiser & Conley 1998). The results of this and previous (Gabai *et al.* 2004) works indicate that the DHEA placental secretion increases in late pregnancy, probably depending upon the tissue mass (Geiser & Conley 1998), and suddenly decreases after parturition, and the lactating mammary gland can affect the circulating levels of DHEA by converting it in other steroids (Belvedere *et al.* 1996). In other physiological conditions, plasma DHEA concentrations are rather stable, and they seem to be slightly altered only in the case of inflammatory events (Almida *et al.* 2005).

For these reasons and because the dairy cow is often affected by inflammatory events during the last 2 months of pregnancy and the first 2 months of lactation (transition period) (Bionaz *et al.* 2007), in our opinion the investigation about the biological role of DHEA in this species should be focused particularly during the peripartum.

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