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# Hair cortisol and dehydroepiandrosterone sulfate concentrations in healthy beef calves from birth to 6 months of age

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## Abstract

Cortisol (C) and dehydroepiandrosterone (DHEA) are recognized as the main fetal steroids, and they are likely to influence fetal development and have long-term effects on newborn hypothalamic-pituitary-adrenal axis (HPA) function. DHEA is often measured as its sulfates and expressed as DHEA-S. Hair analysis represents a promising methodological approach for the non-invasive measurement of steroids, allowing for a retrospective analysis of the total exposure to steroids over time, and avoiding the influence of acute events or circadian fluctuations. Hair cortisol and DHEA concentrations have been investigated in cows, but no studies have been performed on calves. The object of this study was to evaluate hair cortisol (HC) and hair DHEA-S (HDHEA-S) concentrations in beef calves from birth to six months of age. Hair samples of 12 beef calves (seven males, five females) were firstly collected at birth (T1) and then every three weeks up to six months of age (T2-T10), collecting only the re-growth hair. HC and HDHEA-S were analyzed by radioimmunoassay (RIA). Calves sex, weight and APGAR score were registered immediately after birth. Statistical analysis revealed that both HC and HDHEA-S were influenced by sampling time ($P<0.001$). HC concentrations were higher at T1 compared to all subsequent samplings (T2-T10, $P<0.01$); HC

concentrations were higher at T2 compared to T4-T10 (P<0.01), while no further changes were detected from T3 onward. Higher HDHEA-S concentrations were registered at T1, T2 and T3 compared to all the other samplings (P<0.01). No correlation was found between hair concentrations of both steroids and calf sex or birthweight. APGAR score was negatively correlated only with HC at birth (P<0.05). These data demonstrate that C and DHEA-S are quantifiable in the hair of calves and are influenced by their age. The higher HC detected at birth (T1) probably reflects the high serum C concentrations present late in pregnancy and increased by the fetal HPA axis, by which parturition is initiated in cows. The highest HDHEA-S at birth (T1) in calves indicates that the largest amounts of DHEA and its sulfates are produced during fetal development. Moreover, the findings of higher HC at three weeks after birth and of higher HDHEA-S until six weeks after birth, suggest that C and DHEA secretion continues also beyond birth, and that these steroids could be involved in the events occurring during the challenging first weeks of age in the calf.

**Key words**: bovine; calves; cortisol; dehydroepiandrosterone sulfate; hair.

## 1. Introduction

Although steroidogenesis in maternal, placental, and fetal compartments is interdependent, the maternal and fetal hypothalamic-pituitary-adrenal (HPA) axes represent separate biological systems. Among all the hormones involved in the parturition phases, cortisol (C) and dehydroepiandrosterone (DHEA) play a pivotal role. Cortisol is essential for final intrauterine fetal development and maturation [1], as well as to trigger the process of parturition in species like ruminants [2], but also to drive the transition to the extrauterine life [3] which consists in structural and physiological changes from the last intrauterine stage of development until the end of the neonatal period. DHEA is a natural steroid prohormone, and it is a key intermediate in the biosynthesis of biologically potent androgens and estrogens [4]; it is known to exert neuroprotective effects and to play a balancing role against a wide range of C effects [5-6].

In human species, it is becoming increasingly evident that the perinatal period may have a role in defining the healthy outcomes during the adulthood [7]. Long-term consequences of increased fetal exposure to maternal C include increased HPA axis reactivity and susceptibility to neurodevelopmental problems [8], besides a decreased fetal growth [9]. While circulating C levels can result by both the fetal and maternal HPA axis and, in some species, also by the placenta [10], the concentrations of DHEA and its sulfates are higher in the umbilical artery than in the umbilical vein, indicating that these steroids are produced mainly in the fetal compartment [11]. It is clear, therefore, that the study of these hormones might offer a view on HPA axis activity.

Hair analysis represents a consolidated methodological approach for the non-invasive measurement of steroids, allowing for a retrospective analysis of the total exposure to steroids over time, and avoiding the influence of acute events or circadian fluctuations [12]. As a cumulative matrix, hair incorporates hormones and other circulating substances during its growth period [13-15]. The mechanisms by which C incorporates itself into shaved and growing hair were proposed by Henderson [16] using a multiple pool model that included diffusion from blood to the growing follicle, diffusion from the apocrine and sebaceous gland after shaft formation, and absorption from the external environment. Several studies have examined the usefulness of hair cortisol (HC) concentrations for the evaluation of chronic stress in cattle [17-23], and it was concluded that HC concentrations increase in stress situation caused by clinical diseases and are also correlated with pregnancy status. Studies on HC concentrations in calves were conducted to measure the stress levels of calves reared under welfare standards compared to calves reared conventionally [24], and to demonstrate that HC concentrations are not masked by short and non-recurrent moments of stress [25]. However, no studies investigated HC concentrations in the healthy newborn calf immediately after birth. Hair DHEA concentrations have been assessed in adult cows [26-27] but, to the best of the authors' knowledge, not in newborn calves. In cattle, it has been reported that cortisol/DHEA ratio increases in lame dairy cows [26] and following transportation of young bulls [28], but decreases in cows with metritis and leucopenia [29], thus revealing conflicting results when evaluating different

stress conditions. Recently, DHEA has been suspected for growth promoting abuse in cattle, and the measurement of DHEA metabolites have been investigated in calf urine [4], in view of the monitoring programs on hormone residues. The assessment of hair DHEA sulfate (HDHEA-S) concentrations in healthy calves may allow not only to investigate possible alteration due to pregnancy disturbances or to poor welfare conditions, but it may represent also a useful tool in the screening strategy to trace abuses with steroids in this species, especially in beef breeds. The detection of endogenous steroids in fact remains challenging, as concentration-based urinary thresholds may not provide conclusive results due to large inter-individual variations [30-31], so that the use of new biological matrices is under investigation [32-33].

The aim of this study was therefore to assess the concentrations of C and of the main sulfate ester of DHEA (DHEA-S) in the hair of healthy beef calves from birth to six months of age.

## 2. **Materials and methods**

Although hair sampling is a non-invasive procedure, the trial was carried out in accordance with EU Directive 2010/63/EU, and it was approved by the Ethical Committee of the University of Milan (OPBA_146_2019).

### *2.1. Animals*

A total of 12 crossbreed beef calves born by spontaneous delivery were enrolled. Calves sex, birthweight, and APGAR score [34] were assessed immediately after birth. All animals belonged to a single herd in northern Italy. For the first two months after birth calves were housed in individually slatted pens with open partitions that allowed visual, olfactory, and a limited physical contact, and subsequently allocated to group pens. Immediately after birth, calves received colostrum twice a day for the first three days, while from the 4$^{th}$ day after birth they were fed with milk substitute reconstituted at 125 g powder/L. During the study, animals also received a commercial fodder, formulated according to the National Research Council recommendations [35] and wheat straw. Fresh

water was available at automatic drinkers at all time. Feeding management and hygiene was under technician supervision.

*2.2. Hair samples collection*

In the first hair collection at birth (T1), only white hair was taken at the level of the skin with a razor from the animal's forehead, after careful washing and drying of hair coat to remove eventual amniotic and allantoic fluids residuals. At this time, an area of about 10 $cm^2$ was shaved to allow further hair collections. All the subsequent samplings, accomplished every three weeks (T2-T10) until six months of age, were in fact performed collecting only the re-growth hair. At each sampling time individual hair samples were coded and stored in dry tubes at room temperature until analysis.

*2.3. Hair hormone analysis*

The hair strands were placed in polypropylene tubes, covered with isopropanol (Merck KGaA, Darmstadt, Germany) (5 mL), and gently mixed for 3 min at room temperature. The sample was again washed with isopropanol and air dried. This washing procedure minimized the risk of extracting hormones from outside the hair, and it also ensured the removal of dust and any steroids on the surface of the hair sample due to sweat and sebum. Subsequently, 60 mg of trimmed hair was extracted in a glass vial with 3 mL of methanol (Merck KGaA, Darmstadt, Germany) at 37 °C for 16 h. As reviewed by Gao and colleagues [36], methanol is considered the preferential extract as it is able to dissolve neutral, hydrophilic and moderately lipophilic compounds and, given its hydrophilic nature, can penetrate into hair cells and produce swelling of the matrix, thus liberating the enclosed steroids. A downside of extraction by methanol is that it often incorporates interfering substances. While aqueous acids and buffered solutions may yield cleaner extracts than methanol, they may induce hydrolysis thus leading to unwanted loss/decomposition of analytes. Next, in our study, the liquid in the vial was evaporated to dryness at 37 °C under an airstream suction hood. The remaining residue was dissolved in 0.6 mL of phosphate-buffered saline (PBS), 0.05 M, pH 7.5, 0.1% BSA. The concentrations of hair cortisol (HC) [21] and dehydroepiandrosterone sulfate (HDHEA-S) were measured using a solid-phase microtiter radioimmunoassay (RIA) assay. In brief, a 96-well microtiter plate (Optiplate,

Perkin-Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum diluted 1:1,000 in 0.15 mM sodium acetate buffer, pH 9, and the plate was incubated overnight at 4 °C. The plate was then washed twice with RIA buffer, pH 7.5, and incubated overnight at 4 °C with 200 µL of the anti-hormone serum diluted to 1:20,000 for cortisol and 1:800 for DHEA-S. The cross-reactivities of the rabbit anti-cortisol antibody (Analytical Antibodies, Bologna, Italy) with other steroids were as follows: cortisol 100%; cortisone 4.3%; corticosterone 2.8%; 11-deoxycorticosterone 0.7%; 17-hydroxyprogesterone 0.6%; dexamethasone 0.1%; progesterone, <0.01%; 17-hydroxypregnenolone, <0.01%; DHEAS, <0.01%; androsterone sulphate, <0.01%; pregnenolone, <0.01%. DHEA-S was analyzed using a commercial anti-dehydroepiandrosterone sulfate-7ß-CM-BSA (Spi Bio, Montigny Le Bretonneux, France), demonstrating the following cross-reactions: DHEA-S, 100%; 4-androstenedione, 0,2%; testosterone, <0.01%; DHEA, <0.01%. After washing the plate with RIA buffer, standards (5–200 pg/well), a quality control extract, the test extracts, and tracer (cortisol, Perkin-Elmer Life Science, specific activity: 72.4 Ci/mmol, 23 pg/well; DHEA-S, Perkin-Elmer Life Science, specific activity: 55.3 Ci/mmol, 20.3 pg/well) were added in duplicate, and the plate was incubated overnight at 4 °C. The bound hormone was separated from the free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 µL of scintillation cocktail, the plate was counted on a β-counter (Top-Count, Perkin-Elmer Life Science, Boston, MA, USA).

For cortisol, the intra- and inter-assay coefficients of variation (CV) were 3.6 and 9.8% respectively. The detection limit of the assay, as calculated by the software Riasmart (Perkin-Elmer Life Science, Boston, MA, USA), was 24.6 pg/ml.

For DHEA-S, the intra- and inter-assay CV were 3.6 and 12.7%, respectively. The detection limit of the assay, as calculated by the software Riasmart (Perkin-Elmer Life Science, Boston, MA, USA), was 15.8 pg/ml.

To determine the parallelism between DHEA-S standards and endogenous DHEA-S in bovine, hair samples containing high concentrations of endogenous DHEA-S were serially diluted in 0.05 M PBS, pH 7.5. The parallelism between the hair dilution curve and the standard curve indicated that

hair DHEA-S and standard DHEA-S reacted identically with the antibody because a high correlation ($r = 0.99$) was observed between the concentrations obtained and those expected. The relationship between hair DHEA-S concentrations and the standard DHEA-S curve was given by the equation $y = 1{,}005\ x - 0{,}46$.

The recovery test was conducted to evaluate the system response to an increasing amount of DHEA-S standard added to a hair extract with low DHEA-S. The percentage of recovery was determined as follows: [(measured DHEA-S in spiked sample)/(measured DHEA-S in non-spiked sample + DHEA-S added) x 100]. The recovery test revealed a recovery rate of 95.6 ± 6.0% (mean ± SD).

*2.4. Statistical analysis*

Firstly, data were checked for normal distribution by Shapiro-Wilk test, and then statistically analyzed by analysis of covariance (ANCOVA). The effects of the sampling time as fixed factor, and of covariates such as newborn sex (male or female), birth weight and Apgar score on cortisol and DHEA-S hair concentrations were assessed. The Tukey test was used to investigate the effect of each sampling time on hair hormone concentrations. Significance was set at $P<0.05$ (JASP, ver 9 for Windows platform). Both hormone concentrations were expressed as pg/mg of hair.

## 3. Results

All the 12 calves (seven males and five females) were born at a physiologic term of pregnancy and were viable with an APGAR score ≥7 (range 8-10), considered as normal for healthy calves [34]. Birthweight ranged from 40 to 68 kg. The clinical monitoring from birth to six months of age did not record diseases or managerial troubles.

The collection of the hair was easily performed without disturbance in all the animals at all sampling times, and in all cases the collected amount of hair (20-65 mg) was enough to allow the analyses of both hormones.

The profiles of HC and HDHEA-S concentrations in the first six months of age in the 12 calves are reported in Figs. 1 and 2, respectively.

The statistical analysis revealed that both HC and HDHEA-S concentrations were influenced by sampling time ($P<0.001$ for both analytes). Higher HC concentrations were found at T1 compared to T2 and to all the subsequent sampling times ($P<0.01$), and HC concentrations at T2 were higher compared to T4-T10 ($P<0.01$), while no further significative changes were detected from T3 onward. Regarding HDHEA-S, higher concentrations were registered at T1-T3 compared to the subsequent samples (T4-T10) ($P<0.01$). No correlation was found between both HC concentrations and HDHEA-S concentrations, and newborn sex or birthweight. A negative correlation was found between HC concentrations at birth and Apgar score ($P<0.05$).

## 4. Discussion

The collection of hair from calves in the present study allowed to consolidate hair sample as a valuable matrix for the non-invasive investigation of hormones in the bovine species. This procedure can be recommended also in newborns calves since it is easily-performed and with minimum restrain. Furthermore, this procedure allows to investigate hormonal variations with a limited number of samplings compared to blood or salivary analyses.

A recent study on cattle fetuses reported that hair first appears around 160 days of gestation and progresses onwards over the entire body surface [37]; therefore, the hair samples collected from calves at birth reflect hormones accumulation starting from fetal hair appearance [38], and they thus express C and DHEA-S hair incorporation from the $6^{th}$ month of pregnancy until birth. The choice of sampling hair every three weeks after birth was based on the presumed adequate time necessary for hair re-growth, on the will of limiting calf manipulation, and on a reasonable time-interval for hormones measurement. The growth rate of hair in Holstein cows depends on the area of the body, and it is estimated 0.30 mm/day at the shoulder and 0.40 mm/day at the hip [22]. Consequently, three weeks represents a reasonable timespan to collect the amount of hair necessary for RIA analyses.

209 Hair cortisol concentrations registered at birth in the present study (mean 23.7 ± 7.65 pg/mg) were
210 the highest of all the investigated period, although much lower than those detected in 15-days-old
211 female calves (114.5 ± 14.43 pg/mg) [17]; more similar HC concentrations were found in newborn
212 healthy horse foals (mean 56 pg/mg) [39]. The higher values found immediately after birth could be
213 explained by the high serum C concentrations present late in pregnancy in the maternal circulation
214 [40], that are stimulated by the fetal HPA axis, the route by which parturition is initiated in cows [41].
215 Recent data in human species suggest that neonatal hair glucocorticoids (GCs) concentrations are
216 influenced by the third trimester increase in HPA fetal axis activity [42], but evidence is lacking
217 concerning which part of intrauterine regulation is reflected in neonatal hair. With this regard, it has
218 been hypothesized that neonatal hair GCs concentrations reflect or the amniotic-fluid levels [43] or
219 the fetal HPA axis activity [44], but it is conceivable that neonatal hair GCs at birth may reflect a
220 combination of both maternal and fetal GCs [45]. Whatever the origin, HC concentrations in the
221 newborn calves at birth (T1) and at 3 weeks after birth (T2) were greater than in all subsequent
222 samplings, and higher than those found in 3 months old calves [24] and in adult cows [18,22],
223 highlighting the fetal role in steroids production. The finding of lower HC concentrations at 3 weeks
224 of age (T2) compared to birth suggests that during the first three weeks after birth the activity of the
225 HPA axis of the calf is decreased compared to the intrauterine life, and that preparation for birth is a
226 stronger stimulus for the HPA axis than parturition and neonatal adaptation. Nevertheless, it must be
227 remembered that HC concentrations at birth may partially reflect also the C produced by the placenta,
228 while HC concentrations from T2 to T10 merely result from the calf production itself. Although with
229 decreasing values, the higher HC concentrations at 3 weeks of age compared to the subsequent
230 samplings is probably due to the process of birth and to the multi-organ final maturation and neonatal
231 adaptation.

232 In the present study, the mean HC concentrations in calves from 6 weeks to the 6$^{th}$ month after
233 birth, that ranged from 5.8 to 2.0 pg/mg, were comparable with the HC concentrations recently found
234 by Braun et al. [24] in veal calves of the same ages. Other authors previously reported mean

concentrations of 2.5 pg/mg [18] and of 5.7 pg/mg [22] in adult dairy cows, and of 2.3 pg/mg in adult beef cows [46], while others found higher HC concentrations in 2-years-old cows (12.15 pg/mg) [17]. Differences in the color of collected hair [22] or in methodology for HC concentrations analysis [24] can most likely account for some discrepancies between studies; black hair in fact was reported to contain approximately half the concentration of C than white hair in dairy cows [24]. For this reason, only white hair was collected in the present study. Nevertheless, in view of the present results, it is possible to state that the HC concentrations of calves older than 6 weeks of age are mostly comparable to those of adult cows.

No relationship, nor positive neither negative, was found in the study between HC concentrations and birthweight. In humans, no conclusive results have been reported concerning the relationship between maternal C and neonatal outcome at birth, but high HC concentrations during pregnancy have been associated with an increased risk of miscarriage, premature birth, and low weight at birth [47-49]. The lack of correlation in the present study may be due to the fact that all calves enrolled in the study showed a normal birthweight for the species, and no premature calvings were included. Regarding sex differences, HC concentrations in male and female calves did not differ in the present study, consistently with results obtained in other animal species such as lynx [50], grizzly bears [51], caribou and reindeer [52], horse foals [39], and dogs [53]. Some studies on humans reported no sex differences [15, 54], while other studies carried out in older adults found higher HC concentrations in males compared to females [55-56], suggesting that sex difference in HC concentrations may become more pronounced later in life.

Low Apgar scores have been associated with exaggerated cortisol responses after birth and higher serum concentrations [57], so that the negative relationship between HC concentrations at birth and Apgar score found in this study is not surprising.

To the best of the author's knowledge, this study is the first to report the measurement of hair DHEAS concentrations in calves, since only one study has validated DHEAS analysis by RIA in livestock, namely in boars and gilts [58]. HDHEA-S concentrations at birth (T1) were the highest of

the study period, in agreement with a research on humans revealing that the largest amounts of DHEA and its sulfates are produced during fetal development [59], and that this production falls sharply after birth and remains low for several years. In the present study, HDHEA-S remained high until six weeks after birth (T3); it has also been reported a lag time of approximately two weeks for the C deposition in hair, because of its initial deposition in the beneath skin [60], and the same could be hypothesized for DHEA-S. Moreover, during human gestation, DHEA represents the major precursor for oestriol production in the feto–placental unit [61-62], so that the placental conversion represents the major clearance mechanism for DHEA removal in fetus [63]; this conversion is obviously no more possible after birth. Furtherly, results from human studies have shown a persistence of DHEA production by the fetal zone of adrenal cortex also during the postnatal period [64]. Similarly, studies on rhesus monkey showed that, due to the persistence of this fetal zone in adrenal glands in the absence of placental conversion, DHEA levels in the infant are even increased compared to the fetus [63,65-66]. Considering all the aforementioned mechanisms, the finding of high HDHEA-S still six weeks after birth (T3) in calves indicates that parturition and the first weeks of the newborn extrauterine life are characterized by the highest levels of DHEA-S, mostly due to the fact that this steroid is still produced by the adrenal glands of the calf, but no more removed by placenta. Starting from the third month after birth (T4), HDHEA-S in calves decreases and maintains lower stable values until the end of the study (mean range 19.2-33.9 pg/mg).

In the present study, the lack of correlation between HDHEA-S and birthweight and APGAR score, is consistent with data from Fusi et al. [67] on newborn dog puppies. On the contrary, in humans, a negative association between birthweight and serum DHEA-S levels in later childhood has been found [68-70], but this has been linked to genetic or early epigenetic factors that have an impact on adrenal androgen secretion [70]. A study on rhesus monkey found that DHEA is the only hair steroid hormone that clearly differentiated male and female infants [71], with almost double concentrations in female infants. Shen et al. [72] found that the human hair DHEA had a wide range of variation (from 5 to 428 pg/mg) in relation to gender, age and environmental changes. Specifically,

hair DHEA were higher in adult males compared to females and to children. The lack of differences in HDHEA-S between male and female calves in this study may rely on the fact that HDHEA-S was measured from birth forward, thus reflecting the hormones incorporated in the hair towards the last trimester of gestation, whereas sexual differentiation is known to take place even earlier than Day 60 of gestation in cattle [73], far away from the first hair sampling performed in this study, at birth. The lack of sex-related differences in HDHEA-S concentrations agrees with recent data on DHEA-S concentrations in coat and claws of newborn dog puppies [67].

The present results are related only to healthy calves born by spontaneous parturition, and therefore provide information about the normal conditions in newborn calves and young calves. New insights are added about HC concentrations in the calf, while data on HDHEA-S concentrations are, to the best of the author's knowledge, completely new. These results may represent the benchmark for further investigation in calves born by different type of delivery or affected by clinical diseases.

## 5. Conclusions

In conclusion, the present study provides evidence of the usefulness of hair as a valuable and non-invasive matrix for studies on hormonal changes in calves. HC concentrations showed a trend of decrease from birth, reaching stable values after the 6 weeks after birth; HDHEA-S concentrations were higher at birth and at three and six weeks after birth compared to all subsequent samples. These results suggest that C and DHEA-S secretion in the calf continues also beyond birth, and that these steroids could be involved in the events occurring during the first weeks of age.

## Declarations of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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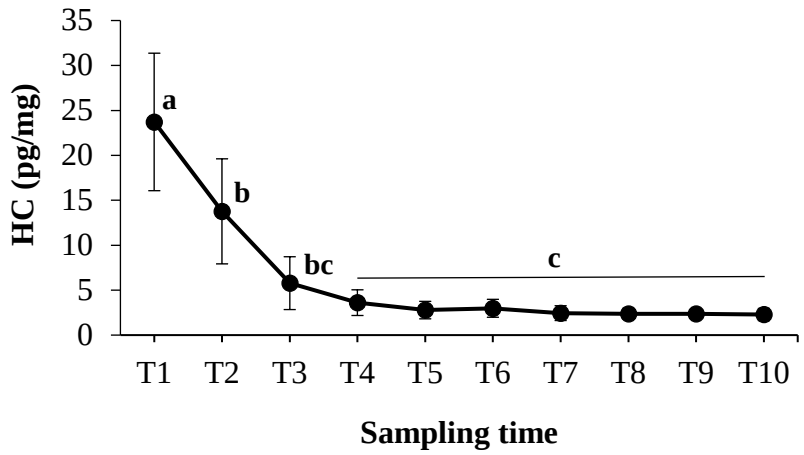
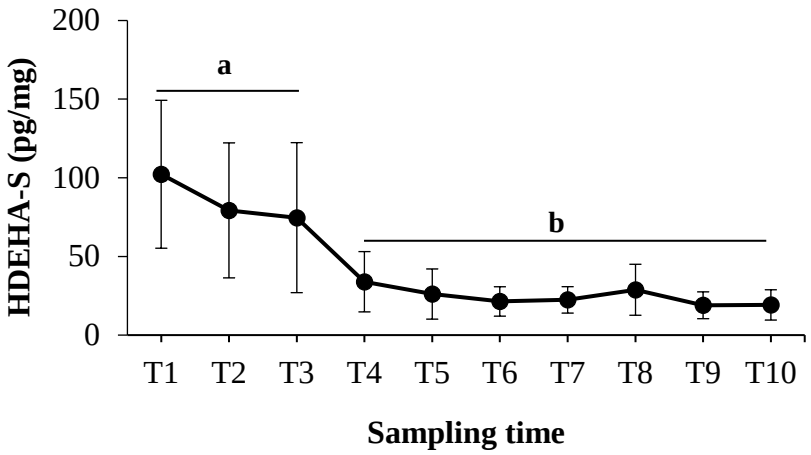



a, b, c: differences between sampling times with $P<0.01$




a, b: differences between sampling times with $P<0.01$

**Highlights**

- The utility of hair matrix for retrospective hormonal changes was assessed in calves
- Cortisol and DHEA-S hair concentrations were investigated from birth to 6 months of age
- A significant effect of age was found on both hormones hair concentrations
- Stable hair C and DEHA-S concentrations were reached 3 and 6 weeks after birth respectively
- Both steroids could be involved in the events occurring in the first weeks of age in calves