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Study of progesterone and cortisol concentrations in the Italian Friesian claw

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ABSTRACT

The present research was conducted to study progesterone and cortisol concentrations in the claw of cattle and to verify whether the cattle claw could be considered an efficient matrix to provide retrospective information regarding progesterone and cortisol concentrations related to pregnancy and peripartum periods. These 2 steroids are involved in hoof growth. The study was performed on 32 calves and 24 pregnant milking cows of the Holstein breed, which were clinically healthy and lacking any claw disorders. Samples of at least 0.5 cm in thickness were taken from the sole. Progesterone and cortisol concentrations were determined by RIA. The cortisol concentration in the horny shoe of calves from 0 to 30 d of age was significantly higher than the concentration at 31 to 60 and 61 to 120 d of age. Conversely, the progesterone concentration showed no statistically significant difference in relation to age. The horn progesterone concentrations recorded in the milking dairy cows at 7 mo of pregnancy showed high variability in the horizontal sections of the sole (the individual coefficient of variation ranged between 0.09 and 1.11). In 6 cows, genuine extreme values (genuine outliers) of the progesterone level were found. Moreover, significant differences existed among the progesterone concentrations of the sole's transverse sections. We detected a significant positive correlation between the weight of the horn samples after freeze-drying and their weight after hydration. The cortisol and progesterone levels in soaked horn samples were found to be significantly lower than in the same dry samples. These results show that cortisol and progesterone can be measured in the cattle claw horn. The claws of mature dairy cows could not be used as a matrix to provide a retrospective measure of cumulative hormone secretion, whereas the analysis of the calves' claw horns showed retrospective hormonal information similar to hair samples.

Key words: claw horn, cortisol, progesterone, dairy cow

INTRODUCTION

The current literature lacks information regarding steroidogenic functions of the claw horn. Cortisol synthesis in the skin (melanocytes and keratinocytes) has been previously described (Ito et al., 2005; Slominski et al., 2005; Slominski et al., 2006), yet it remains an open question whether the cortisol concentration in hair reflects the cortisol concentration in the peripheral circulation or from local production (or both). Currently, very little information is available about the factors that influence the rates of hoof growth. Several studies suggest that hormones play a critical role in the normal development of the claw horn and proper keratin formation (Tomlinson et al., 2004). In particular, Hendry et al. (1999) found that glucocorticoids affect the maturation of keratinocytes through regulation of protein synthesis; cortisol inhibited keratin protein synthesis in bovine hoof-tissue explants (Hendry et al., 1999). Epidemiologists have yet to identify a causative relationship between systemic glucocorticoid concentration and laminitis in dairy cows, resulting from the formation of an inferior claw horn (Goff and Horst, 1997). Instead, progesterone, a steroid hormone elevated in pregnancy, has been shown to have antiinflammatory and immunosuppressive properties as well as to increase keratinocyte proliferation (Urano et al., 1995). Although progesterone is generally known as a reproductive hormone released by the gonads, it is also produced in the brain and by the adrenal gland, where it is an indirect precursor to cortisol (Baulieu et al., 2001).

Claw quality is the product of horn characteristics, claw shape, and the anatomy and physiology of the inner structure (Politiek et al., 1986). Some authors (Vermunt and Greenough, 1995) define high claw quality as a low susceptibility to claw disorders, resulting in a lesser need for claw care and fewer economic losses. Hoof disorders, behind mastitis and reproductive failure, reduce animal health and contribute to economic losses for farmers (Whitaker et al., 1983). Costs include treatment, decreased milk production (Sogstad et al., 2007), decreased reproductive performance, and increased culling (Sogstad et al., 2006). Italian Friesian

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cows are generally subjected to frequent biological and environmental changes and are susceptible to claw disorders during their long productive lives (Sogstad et al., 2007; Brizzi, 2008).

Fingernail analysis in human studies allows for the assessment of accumulated hormone levels over a longer time period, as neutrally charged endogenous hormones passively diffuse from the capillaries to the nail matrix and become incorporated into the keratin during nail formation (de Berker et al., 2007; Warnock et al., 2010). In fact, several studies support the use of human nails as a matrix for the measurement of cortisol (Raul et al., 2004; Khelil et al., 2011) and for retrospective information such as hair samples (Comin et al., 2013; Peric et al., 2013). No one has ever measured the steroid levels in the claws of even-toed ungulates; studies of this matrix have been limited to understanding the horn tissue's inner composition (de Berker et al., 2007) and the biomechanical unbalancing of bovine claws (Toussaint Raven, 1999).

The present research was conducted using a noninvasive method to study progesterone and cortisol concentrations in the claw of cattle to verify whether the claw (used to walk and highly mechanically loaded more or less homogeneously with BW) of an artiodactyl species such as the cow, could be considered an efficient matrix to provide retrospective information regarding progesterone and cortisol concentrations related to pregnancy and peripartum periods.

MATERIALS AND METHODS

Although hoof sampling is a noninvasive procedure, the study was carried out in accordance with the European Union Directive 2010/63/EU for animal experiments. To avoid stress and in compliance with current legislation on animal welfare, the samples were collected during a claw-trimming procedure.

Animals and Horn Samples

The experiment was performed on single horn samples from 32 calves (8 calves up to 30 d old, 13 from 31 to 60 d old, and 11 from 61 to 120 d old) and 24 milking Friesian cows at 7 mo of pregnancy, clinically healthy and lacking any claw disorders. The calves were maintained in individual pens, outside the principal shed. The cows were housed in freestalls, bedded with sawdust, and on slotted floors in the feeding area.

With the support of a trained hoof trimmer, the hooves of calves and cows were pared to reveal a clean horn surface, and samples of the soles were taken, sealed into tubes to maintain them in an unaltered condition, and stored at −20°C until use. The samples were taken from the fore claws because they are more stable than the hind claws; they generally show fewer foot diseases and remain healthier (Toussaint Raven, 1999). During the claw-trimming procedure, the cows were locked into the claw treatment crush, and the sole of the front right hoof was divided into 4 areas, using different colors to identify the area of origin for the horizontal samples taken by the podiatrist with an electric cutter. These samples, all of the same thickness (maximum of 1 mm), were immediately organized according to color (related to the area of origin) and sealed into tubes to prevent alteration. After the trimming procedure, a horn thickness of 0.5 to 0.7 cm was always maintained in the anterior part of the sole (Toussaint Raven, 1999), and all samples taken from the sole were at least 0.5 cm in thickness. In addition, in a group of 6 dairy cows randomly selected from the 24 dairy cows considered, 8 sequential transverse sections were taken from the same claw, from the sole to the coronet of the claw (S0, S1, S2, S3, S4, S5, S6, S7, and S8), each 420 μm thick, as measured using an automatic microtome (Automatic Microtome MR-3 RMC/Boeckeler; Boeckeler Instruments Inc., Tucson, AZ). Because the sole of the claw thickens by 4 to 5 mm each month (da Silva et al., 2010), the 420-μm transverse sections obtained with the microtome represent a horn growth of 2 or 3 d.

Freeze-Drying

All of the samples were freeze-dried (Heto Freeze Drying Hetossic CD4; Heto Holten (UK) Ltd., Surrey, UK) as described by Damion et al. (2012) and the dry weights were calculated.

Hydration

Horn samples from the right front claw of all the cows were assigned to a soaking treatment in PBS, as described by Winkler and Margerison (2012). The soaked digits were completely immersed in Petri dishes for 72 h. At the end of the soaking period, each segment was weighed. These samples were then freeze-dried for 24 h and finally weighed to determine their dry weights.

Horn Cortisol and Horn Progesterone Assay

The dried horn was extracted with 3 mL of methanol per 30 mg of trimmed horn section for 16 h at 37°C. Next, 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 PBS (0.05 *M*; pH 7.5). A recovery test on 5 replicates was performed by adding 125, 250, 500, or 1,000 pg of ^{3}H-cortisol or ^{3}H-progesterone (PerkinElmer Life Sciences Inc., Boston, MA) to 30 mg of trimmed horn and incubated for 16 h at room temperature. The extraction was performed as described above. The mean percentage recovery was 89.05 ± 2.68 and 87.98 ± 2.34%, respectively, for cortisol and progesterone.

The concentration of cortisol and progesterone in all of the samples was measured using a solid-phase microtiter RIA as described by Comin et al. (2011). In brief, a 96-well microtiter plate (OptiPlate; PerkinElmer Life Sciences Inc.) 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 7.4) and incubated overnight at 4°C with 200 μL of the antibody serum diluted 1:12,000 for cortisol and 1:8,000 for progesterone. The rabbit anti-cortisol antibody used was obtained from Biogenesis Ltd. (Poole, UK). The cross-reactivity of the anti-cortisol antibody with other steroids was as follows: cortisol, 100%; corticosterone, 1.8%; and aldosterone, <0.02%. The cross-reactivity of the anti-progesterone antibody with other steroids was as follows: 11 β-OH-progesterone, 46%; 17 α-OH-progesterone, 0.4%; 20α-OH-progesterone, 0.04%; testosterone, 0.08%; cortisol, <0.01%; estradiol 17β, <0.01%; estradiol 17α, <0.01%; and estrone, <0.01%. After washing the plate with RIA buffer, standards (5–300 pg/well), a quality-control extract, the test extracts, and tracer (hydrocortisone {cortisol [1,2,6,7-^{3}H (N)]-} and progesterone [1,2,6,7-^{3}H (N)]; PerkinElmer Life Sciences Inc.) were added, and the plate was incubated overnight at 4°C. Bound hormone was separated from free hormone by decanting and washing the wells in RIA buffer. After addition of 200 μL of scintillation cocktail, the plate was counted on a β-counter (TopCount; PerkinElmer Life Sciences Inc.). For cortisol, the intra- and interassay coefficients of variation were 3.7 and 10.1%, respectively. The sensitivity of the assay, calculated as the interpolated dose of the response to a concentration of zero minus the statistical error, was 1.23 pg/well. For progesterone, the intra- and interassay coefficients of variation were 3.4 and 8.2%, respectively. The sensitivity of the assay, calculated as the interpolated dose of the response to a concentration of zero minus the statistical error, was 0.56 pg/well. The progesterone and cortisol concentrations are expressed in picograms per milligram of dry weight.

Statistical Analysis

The statistical analysis was performed using SPSS for Windows (v. 7.5.21; SPSS Inc., Chicago, IL). The normality of the data distribution and homogeneity of the variance were tested using the Kolmogorov-Smirnoff and Levene's tests, respectively. Where appropriate, the data were logarithmically transformed for parametric testing. A one-way ANOVA was generated to determine the main effects of age on the cortisol and progesterone levels of the sole of the calves. The Tukey-Kramer test for unequal sample size was used as a post hoc test. The effect of the different sampling areas in the horizontal and vertical sections on the progesterone level was evaluated by ANOVA, considering the sampling area and the animal as the fixed effect and block, respectively. For multiple comparisons, the Bonferroni adjustment was made. For the progesterone level, the analysis of genuine extreme values within each animal was performed using the extreme studentized deviate test and using Grubbs' test. The coefficient of variation was calculated according to Sokal and Rohlf (1995). The correlation between the dry and soaked weights of the samples was determined by Pearson's test. A paired-samples t-test was used to evaluate the cortisol and progesterone concentrations in splinters before and after the soaking period.

RESULTS

The cortisol concentration in the horny shoe of calves at 0 to 30 d of age was significantly higher ($P < 0.05$) than the concentrations found at 31 to 60 and 61 to 120 d of age (Figure 1). In contrast, the progesterone concentration did not show any statistically significant difference in relation to age ($P > 0.05$; Figure 2).

In the cow claws, the concentration of progesterone in the different sole areas (A, B, C, and D) was not significantly different. However, an animal effect was observed ($P < 0.01$; Table 1). The horn progesterone concentrations recorded in the dairy cows ranged from 14.32 to 103.66 pg/mg. For each individual animal, variability was found between the areas belonging to the same claw with a coefficient of variation that ranged between 0.09 and 1.11. In particular, 12 animals

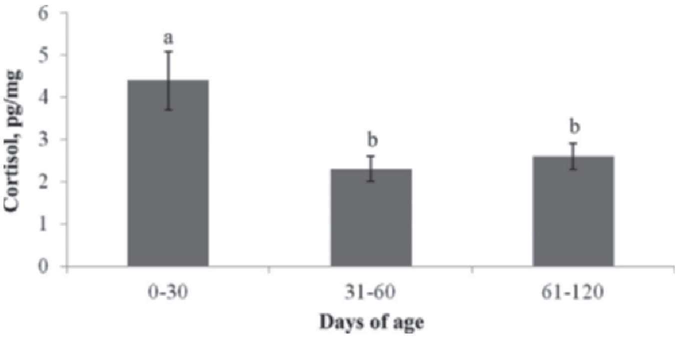

Figure 1. Horny shoe cortisol concentration in calves at 0 to 30, 31 to 60, and 61 to 120 d of age. The data are expressed as the mean value ± SE. Different letters (a and b) indicate a statistically significant difference ($P < 0.05$).

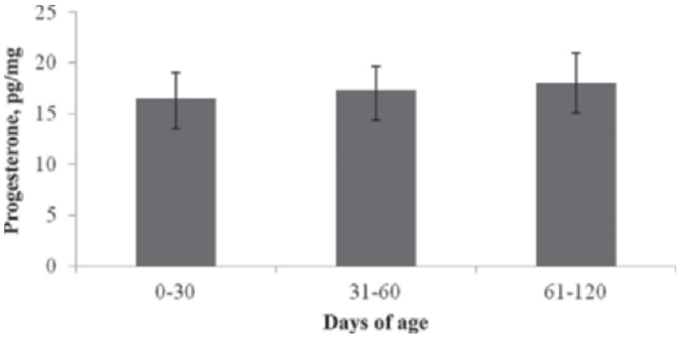

Figure 2. Horny shoe progesterone concentration in calves at 0 to 30, 31 to 60, and 61 to 120 d of age. The data are expressed as the mean value ± SE.

presented a coefficient of variation lower than 0.40, 11 lower than 0.8, and 2 animals had a coefficient of variation higher than 0.8. Moreover, 6 animals out of 24 showed genuine extreme values. This finding indicates that the progesterone value in some areas was numerically distant from the others. Therefore, the horizontal variability is independent of the sampling area but is subject to the animal's individual reaction.

The results recorded in Table 2 show significant differences between the progesterone concentrations of the transverse sections of the sole ($P < 0.01$) from the distal (S0) to proximal sections (S8). Moreover, a significant effect on the animal was also observed ($P < 0.01$). The progesterone levels significantly decreased from the outside to the inside of the sole (from S0 to S8).

A significant positive correlation was found ($r = 0.99$; $P < 0.001$) between the weight of the horn samples after freeze-drying and their weight after hydration. The cortisol and progesterone levels in the soaked samples of horn were found to be significantly lower than in the same dry samples ($P < 0.01$ and $P < 0.05$ for cortisol and progesterone, respectively; Figure 3).

DISCUSSION

This research deepens our knowledge about steroid concentrations in horn tissue. Warnock et al. (2010) used this matrix to pilot the measurement of hormone concentration (cortisol and dehydroepiandrosterone) in young students in relation to environmental stresses, as a potential measure of the accumulated secretion of steroid hormones over a prolonged time period. To date, no measurements of steroids in the claws of even-toed ungulates exist. The horn samples from the calves seem to suggest that the soles of the claws of calves are similar to human nail and hair samples; they demonstrated, in fact, the same memory effect (McMillen et al., 1995; Gow et al., 2010; Fourie and Bernstein, 2011). The cortisol and progesterone concentrations in the horn samples of calves follow the same trend as samples of hair from calves and foals during the peripartum period (Comin et al., 2008, 2012). The decrease in cortisol concentration in the horn samples from calves was in agreement with results obtained from Comin et al. (2008) examining hair cortisol levels of calves at parturition and at least 6 mo old. A similar trend of decreasing cortisol levels was also detected in foal hair samples from birth to 90 d of age (Montillo et al., 2012). Possible explanations for this decrease in cortisol levels include (1) the end of the fetomaternal relationship that occurred through the placenta and (2) the progressive

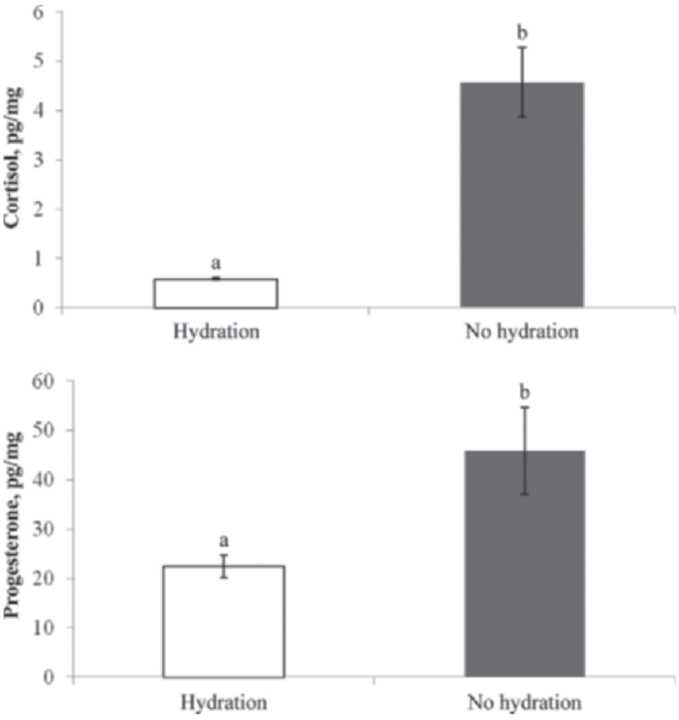

Figure 3. Cortisol and progesterone levels in the soaked and dry samples of mature cow horn from the right claw. The data are expressed in mean value and ± SE. Different letters (a and b) indicate a statistically significant difference ($P < 0.05$).

Table 1. Progesterone (P4) concentrations of varied sole areas

	Horizontal area					*P*-value	
Concentration	A	B	C	D	SEM	Area	Animal
P4, pg/mg	36.9	39.77	28.07	33.33	2.213	NS	<0.01

Table 2. Progesterone (P4) concentrations at the sole's transverse section

	Vertical area[1]										*P*-value	
Concentration	S0	S1	S2	S3	S4	S5	S6	S7	S8	SEM	Area	Animal
P4, pg/mg	52.44^{a}	42.01^{ab}	42.9^{ab}	30.04^{bc}	30.46^{bc}	22.31^{c}	21.87^{c}	19.75^{c}	17.34^{c}	1.338	<0.01	<0.01

$^{a–c}$Different superscript letters indicate statistically significant differences for the area's effect ($P < 0.05$).

[1] S0 to S8 represent 8 sequential transverse sections taken from the same claw, from the sole to the coronet of the claw.

adaptation to extrauterine life. The progesterone profile in a calf's hoof is similar to that in a foal's hair. In fact, Montillo et al. (2012) did not find any significant differences in the progesterone concentrations during the perinatal period. The progesterone measured in the horn tissue is either produced by the adrenal gland or it can have an exogenous origin, mainly via milk nutrition (no calves were fed milk replacer or formula). In calves, these horny matrices, both hair and claw, show similar incorporation capacity and conservation of retrospective hormonal information.

The memory effect was not found in cow claws. It is important to consider that even-toed ungulates use their claws to walk and are thus highly mechanically loaded with BW. This aspect is less significant in a calf compared with a milking adult cow. The results from this study showed that the claw of mature cows cannot be used to determine retrospective information as is done with human nail and hair samples. Despite similar progesterone concentrations between the horizontal areas, the cow claws showed high individual variability, with 6 of the 24 animals presenting genuine extreme values. This means that the incorporation of progesterone in the different areas of the same claw was inhomogeneous, which could be due to a differential response to mechanical stimulation of the hoof. Variability was also demonstrated by analysis of the transverse sections, where the progesterone levels decreased from the distal to proximal sections. These data were unexpected because progesterone plasma levels are high and constant at pregnancy (Bradford, 1972; Astiti and Panjaitan, 2013). This unexpected variability of progesterone levels could be because dairy cows, similar to all even-toed ungulates (Artiodactyla), use their claws to walk, and high mechanically loaded areas of the claw present more growth (Brizzi, 2008). This specific pressure, caused by an uneven load, produces an increased blood supply that could induce higher steroid incorporation. Therefore, unlike hair and human nails, the claws of mature dairy cows cannot be studied as a matrix to provide retrospective hormonal information.

The hydration test demonstrated that the samples' dry weights were positively correlated with their hydrated weights. Several researchers, using different experimental conditions (lower hydration period and a different drying system), have reported that the hydration capacity of claws in a farm varied from 29 to 35%. The bovine claw behaves like a sponge, being 1,000 times more porous than skin (Higuchi et al., 2003; Borderas et al., 2004); it absorbs and releases water in relation to the environment in which it is located, demonstrating an active interface between the claw and the environment. Lower water content makes the horn harder and less prone to hoof diseases or erosion of the horn tissue (Huang et al., 1995; Vermunt and Greenough, 1995; Higuchi et al., 2003). We have found a reduction in the cortisol and progesterone concentrations in horn samples after hydration. In particular, the cortisol concentration decreased 8 fold, whereas the progesterone concentration halved after 72 h of hydration. This reduction could be due to different molecular polarities. Reduction in cortisol and progesterone levels after hydration could alter the claw composition of the horn tissue. It would be interesting to know if hydration could also lead to a deterioration of mechanical characteristics over time.

CONCLUSIONS

This sampling method was not invasive because it took place during the claw-trimming procedure. The claws of calves behaved like hair samples in that both reveal a retrospective image of accumulated steroid hormones. This is most likely because calves lack hoof disorders that arise from unbalanced mechanical loads, in addition to nutritional and environmental imbalances. Unlike hair and human nails, the claws of mature dairy cows cannot be used as a matrix to provide a retrospective measure of cumulative hormone secretion, even if the origin of progesterone and cortisol in the claw is likely similar to the human nail. Moreover, in the claws of mature dairy cows, hormonal concentrations can be altered when exposed to water. The analysis of progesterone concentrations in the sequential transverse sections, which each represented horn growth of 2 or 3 d, showed unexpected variations despite a constant blood level during pregnancy. An unbalanced mechanical load can be taken into account to explain the variations of the steroids studied in the transverse sections. The hoof sequential transverse sections of the mature dairy cow does not register information as observed in hair by Kirschbaum et al. (2009), who analyzed cortisol level variations in sections of hair from a mother who had recently given birth. As a metaphor, the claw is not like hair that behaves as a fly data recorder but can be considered as a monitor with the units as pixels. A claw horn is composed of interjected and independent units that collectively contribute to the creation of all the morphological and functional characteristics of the claw.

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