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Postnatal and postweaning endocrine setting in dairy calves through hair cortisol, dehydroepiandrosterone and dehydroepiandrosterone sulphate

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Abstract

Importance of the work: The care of calves on dairy farms between birth and weaning can improve their long-term development and growth. In fact, a poor newborn health status and a high allostatic load may adversely affect development in dairy cows. To determine cortisol, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEA-S) individually is useful for an understanding of the individual state, being biomarkers of hypothalamic-pituitary-adrenal (HPA) axis activity.

Objectives: As a preliminary study, to investigate the hair concentrations of cortisol, DHEA, DHEA-S and their ratios in dairy calves in two key periods of their growth characterized by considerable environmental changes.

Materials & Methods: Hair sampling was conducted on clinically healthy dairy calves during the postnatal period at age 64.8±0.65 d (POP; mean±standard error; n = 73) and during the postweaning period at age 155.3±0.85 d (PWP, n = 62). The hair hormone concentrations were measured using a radioimmunoassay.

Results: Hair cortisol concentrations were higher in the POP than in the PWP. Furthermore, the cortisol:DHEA and cortisol:DHEA-S ratios were higher in the first period of evaluation, showing a higher animal allostatic load at birth.

Main finding: Identification was achieved non-invasively of calves with a high allostatic load through biomarkers of HPA axis activity. The evaluation of this activity is very important given its influence on many biological processes, such as energy balance, development of the reproductive system and immune response.

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Introduction

The care of newborn animals on dairy farms in the time between birth and weaning can improve the long-term development and growth of calves. In fact, a poor newborn health status, together with other factors, such as improper handling or inadequate facilities and feeding, may adversely affect the development of dairy cows (Bazeley et al., 2016). In high-production dairy farms, the calves are separated from their mothers within 24 h of birth (Broom and Leaver, 1978). The allostatic load related to the separation procedure from the mother and the perinatal period can expose the newborn calf to environmental and social stressors that act simultaneously and, at times, in a sequential way (Enriquez et al., 2011). These can alter homeostasis, thus compromising the immune defenses and, potentially, lead to a poor health status, which can negatively influence the animal's future productive life (Weary et al., 2008; Van de Stroet et al., 2016). These environmental changes and stressors in their various forms, durations and intensities during the postnatal period can induce dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and epigenetic alterations (Lee and Sawa, 2014; Burns et al., 2018). This can lead to a consequent impairment of the biorhythms and a hormetic response of steroid hormones (Chung et al., 2011), resulting in pathological conditions (Nicolaides et al., 2014). The endpoint of the HPA axis is cortisol, which regulates many physiological processes, such as energy balance, the development of the reproductive system and immune and stress responses (Hill and McEwen, 2010).

Cortisol has long been considered a marker of the allostatic load, which is correlated with the body's attempt to adapt to environmental changes (Mormède et al., 2007; Burnett et al., 2014). Cortisol is usually determined in blood samples (Cook et al., 2000; Negrao et al., 2004), saliva (Negrao et al., 2004), urine (Hay and Mormède, 1998), faeces (Möstl and Palme, 2002) and milk (Verkerk et al., 1998). In these matrices, the concentrations of this steroid hormone reflect punctual changes in the circadian rhythm, the diet or stress before sampling. The advantage of using hair is that it provides an integrated measure of hormone concentrations over medium and long periods (Meyer and Novak, 2012), it can be simply and non-invasively collected and it does not require any special storage (Wright et al., 2018).

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEA-S) are hormones related to resilience and the allostatic load (Charney, 2004; Whitham et al., 2020). They are primarily secreted by the zona reticularis of the adrenal glands and act as prohormones for sexual steroids in both males and females. They also have anti-glucocorticoid qualities, presumably as competitive inhibitors of cortisol (Hazeldine et al., 2010). These steroid hormones act on multiple levels, playing a role in immune system activation and they have anti-inflammatory effects, antioxidant properties and are involved in lipid metabolism. In humans, Charney (2004) reported positive associations between plasma DHEA concentrations and adaptation to extreme stress. Russo et al. (2012) indicated a possible relationship between a high concentration of DHEA and positive coping. Because of the opposing effects of DHEA and cortisol, a common measure applied to test the impact of both hormones simultaneously is the ratio between them (Hechter et al., 1997; Goodyer et al., 1998; Qiao et al., 2017). Studies in lame cows demonstrated a decrease in serum DHEA and a higher cortisol:DHEA ratio compared to clinically healthy cows (Almeida et al., 2008). In humans (Shen et al., 2009a; Chen et al., 2013; Gao et al., 2013), cows (Peric et al., 2017), horses (Placci et al., 2020), pigs (Trevisan et al., 2017; Bergamin et al., 2019) and guinea pigs (Shen et al., 2009b), the DHEA hair sample assay has been investigated, but, to date, no study has been published on hair DHEA concentrations in calves. In fact, DHEA-S in calf hair was first described by Probo et al. (2021).

Currently, the emphasis on efficiency, sustainability, welfare and production quality in livestock breeding is leading to new approaches in animal management and monitoring. Among the latter, sensor-based technology is expected to play a pivotal role. Nevertheless, in spite of the latest scientific advancements, there remains a gap between the technological measurement of signals, behaviors and the processes of physiological adaptation of farm animals (Neethirajan, 2020). Hence, "traditional" determination of hormone concentrations remains a valid manner for measuring the physiological status of farm animals; additionally, the new approach relies on the use of specimens non-invasively collected.

Thus, the current study aimed to test the hypothesis that cortisol, DHEA and DHEA-S hair concentrations are different in the perinatal period and in the postweaning period at age 5 mth, when weaning and adaptation to extrauterine life must be already accomplished. Finding differences between them would be important to evaluate variations in the allostatic load and resilience in this species using a non-punctual and non-invasively collected specimen, such as hair, which is not influenced by acute HPA axis stimulations.

Materials and Methods

Ethics statements

Although hair sampling is a non-invasive and unproblematic procedure, the study was carried out in accordance with EU Directive 2010/63/EU (2010) on the protection of animals used for scientific purposes and Italian legislation on animal care (DL n. 26, 04/03/2014) (2014).

Animals

The trial was conducted with 142 Italian Friesian female calves reared on one farm with an intensive dairy production system. Within 3 h of birth, the colostrum was administered and calves were transferred to individual pens where they remained until aged 7 d; they were fed with bulk milk. After this period, the animals were moved to a first multiple box (with an area of 800 m^2) in which they were kept until weaning. The nutrition planning included the controlled administration of bulk milk (through automatic suckles that have the ability to recognize individual animals) supplemented by commercial, pre-weaning, concentrated pellets offered *ad libitum*. The weaning of the calves took place when they were aged between 63 d and 80 d. Then, the weaned calves were transferred to a second multiple box (with an area of 800 m^2) in which they were fed with hay *ad libitum* and with a concentrate composed of 0.6 kg of corn and 0.4 kg of soybean meal. The diets were formulated to meet the nutritional requirements of animals in agreement with the standards stipulated by Institut National de la Recherche Agronomique (2010). All animals were in good condition as verified by the official veterinarian, except for seven traumatized calves (TRA) that, remained blocked in the feed fence for a few hours due to a mechanical problem following the move from the first to the second box.

Hair sampling and animal weighing

White hair samples were collected from the scapular region of 73 calves randomly selected during the postnatal period at weaning (POP; aged 64.8±0.65 d; mean±SE) and during the postweaning period, both from healthy calves (PWP; aged 155.3 ± 0.85 d, $n = 62$) and from TRA (aged 158.9±2.58 d, $n = 7$).

The hair segments in which cortisol is incorporated require time to emerge because the hair cells that take up the hormones are part of the hair follicles beneath the skin. On the other hand, this kind of cells start to capture systemic hormonal concentrations soon after the hair follicle has been developed in the fetus (Kapoor et al., 2016). Considering both the lag time in hair availability above the skin level and the intrauterine development of hair, the first hair sample collected in this study represented the cortisol concentrations characterizing the fetal period and approximately the first 45–50 d after birth.

The second hair sample was taken at about age 155 d, which corresponded to the hormone concentrations characterizing the postweaning period because approximately 4 mth are required for a complete change of hair; during shedding, almost all mature hairs are lost from the skin follicles (Hayman and Nay, 1961).

The hair samples were collected from the withers by shaving close to the skin using an electric razor. This area was chosen as the cleanest and most easily accessible. The hair samples were stored in paper envelopes in the dark at room temperature until the end of the study.

Each calf was weighed at birth and before hair collection.

Hair hormonal assay

The hair strands were washed and extracted, as described in Peric et al. (2013). The concentrations of hair cortisol (Peric et al., 2013), DHEA and DHEA-S (Probo et al., 2021) were measured using a solid-phase microtiter radioimmunoassay (RIA). For DHEA, a 96-well microtiter plate (Optiplate; Perkin-Elmer Life Science; Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum diluted to 1:1,000 in 0.15 mM sodium acetate buffer, pH 9 and the plate was incubated overnight at 4 °C. Then, the plate was washed twice with RIA buffer, pH 7.5 and incubated overnight at 4 °C with 200 µL of the anti-hormone serum diluted to 1:2,000 for DHEA. The rabbit anti-DHEA antibody used was obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and showed the following cross-reactions: DHEA, 100%; pregnenolone, 0.1%; androstenediol, 0.08%; dihydrotestosterone, 0.05%; sulphate DHEA, 0.02%; testosterone, < 0.01%; 5α-androstane-diol-3β,3α, < 0.01%; 5β-androstane-3α, < 0.01%; estradiol, < 0.01%; progesterone, < 0.01%; estrone, < 0.01%; and cholesterol, < 0.01%. After washing the plate with RIA buffer, standards (5–200 pg/well), a quality control extract, the test extracts and tracer (DHEA; Perkin-Elmer Life Science; specific activity: 70.5 Ci/mmol, 15 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 DHEA, the intra- and inter-assay coefficient of variation (CV) were 4.3 and 10.1%, respectively. The detection limit of the assay, as calculated using the software Riasmart (Perkin-Elmer Life Science; Boston, MA, USA), was 8.2 pg/mL.

To determine parallelism between DHEA standards and endogenous DHEA in bovine, hair samples containing high concentrations of endogenous DHEA were serially diluted in 0.05 M phosphate-buffered saline (PBS), pH 7.5. There was a linear relationship between the hair DHEA concentrations and the standard DHEA curve, determined through linear regression, with a correlation coefficient (r) of 0.99 and the model was described by the equation $y = 0.838x + 1.812$.

The recovery test was conducted to evaluate the system response to an increasing amount of DHEA standard added to a hair extract with low DHEA. The percentage of recovery was determined as: [(measured DHEA in spiked sample) / (measured DHEA in non-spiked sample + DHEA added) × 100]. The recovery test revealed a mean (± SD) recovery rate of 97.3 ± 4.8%.

Statistical analysis

The statistical analysis was carried out using R software version 3.4.1, (R Core Team, 2017). Data were analyzed using a boxplot and their normal distribution was evaluated using the Shapiro-Wilk test. The probability density functions to assess the hormone distribution were obtained with the package fitdistrplus (Delignette-Muller and Dutang, 2015). The density functions of normal, log-normal, logistic, exponential and gamma were tested. The most appropriate density functions were selected based on the Akaike Information Criterion (AIC; Burnham and Anderson, 2010).

The differences in hormone concentrations between groups of animals were analyzed using the Kruskal-Wallis test; for the multicomparisons, the Mann-Whitney test was applied. This procedure is the nonparametric analog to one-way ANOVA followed by Fisher's LSD as a post-hoc test (Lin and Haseman, 1977).

Results

Boxplots of cortisol, DHEA and DHEA-S are shown in Figs. 1–3, respectively.

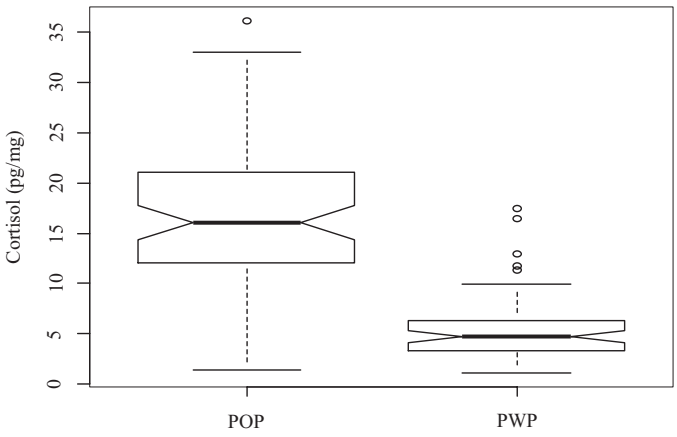

Fig. 1 Hair cortisol concentrations in postnatal (POP) and postweaning (PWP) periods of calves

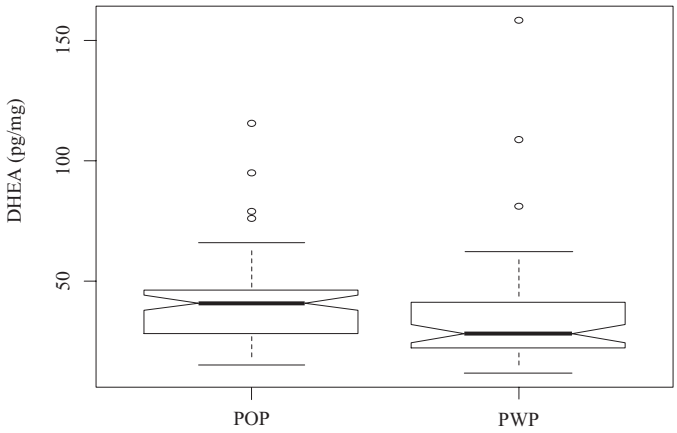

Fig. 2 Hair DHEA concentrations in postnatal (POP) and postweaning (PWP) periods of calves

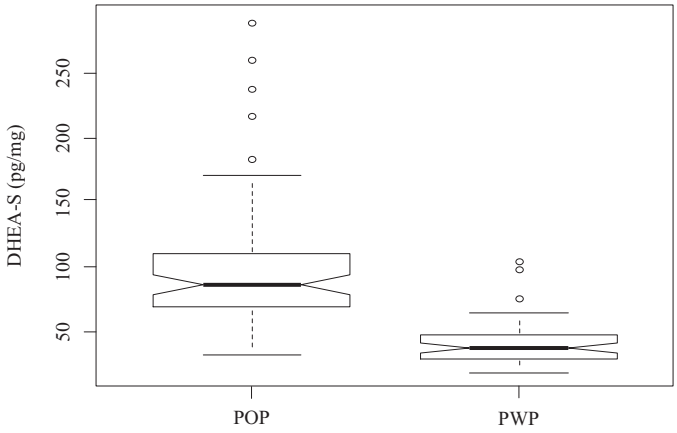

Fig. 3 Hair DHEA-S concentrations in postnatal (POP) and postweaning (PWP) periods of calves

The hair cortisol concentrations recorded in the POP had a normal distribution; however, the logistic distribution can be considered to have substantial support since the ΔAIC was lower than 2 (ΔAIC = 1.02; Burnham and Anderson, 2004). Conversely, hair cortisol concentrations recorded in the PWP had a log-normal distribution. Values regarding both distributions are described in Table 1.

Hair DHEA and DHEA-S concentrations followed log-normal distributions both in the POP and the PWP. Values regarding DHEA and DHEA-S distributions are described in Table 2 and Table 3, respectively.

The average weights of the animals at birth, POP, PWP and TRA hair sampling were 37.6±0.36 kg (n = 142), 89.1±1.04 kg (n = 73) and 176.7±1.52 kg (n = 62) and 179.4±3.50 kg (n = 7), respectively (data not reported in Tables). The average daily gains from birth to sample collection were 0.797±0.011 kg/d, 0.895±0.005 kg/d and 0.881±0.007 kg/d for the POP, PWP and TRA, respectively (data not reported in Tables). Interestingly, the average daily gain of the PWP was similar to that of the TRA ($p > 0.05$; data not reported in Tables). The comparisons between hair hormones in POP, PWP and TRA are reported in Table 4.

Table 1 Best fitting probability density functions to hair cortisol distribution in postnatal and postweaning periods of calves

Function	Parameter 1 (± SE)	Parameter 2 (± SE)	AIC
Postnatal period			
Normal	Mean: 16.89 (0.85)	SD: 7.26 (0.60)	500.57
Logistic	Location: 16.62 (0.84)	Scale: 4.14 (0.40)	501.99
Gamma	Shape: 4.36 (0.70)	Rate: 0.26 (0.04)	504.53
Log-normal	Mean: 2.71 (0.06)	SD: 0.55 (0.05)	518.02
Exponential	Rate: 0.0592 (0.0069)	-	560.66
Postweaning period			
Log-normal	Mean: 1.58 (0.06)	SD: 0.50 (0.05)	290.31
Gamma	Shape: 3.98 (0.69)	Rate: 0.72 (0.13)	295.35
Logistic	Location: 5.06 (0.34)	Scale: 1.53 (0.17)	310.34
Normal	Mean: 5.53 (0.40)	SD: 3.15 (0.28)	322.22
Exponential	Rate: 0.1808 (0.0230)	-	338.08

AIC = Akaike Information Criterion

Table 2 Best fitting probability density functions to hair dehydroepiandrosterone distribution in postnatal and postweaning periods of calves

Function	Parameter 1 (± SE)	Parameter 2 (± SE)	AIC
Postnatal period			
Log-normal	Mean: 3.63 (0.05)	SD: 0.40 (0.03)	607.35
Gamma	Shape: 6.25 (1.01)	Rate: 0.15 (0.03)	610.34
Logistic	Location: 38.88 (1.76)	Scale: 8.85 (0.88)	618.52
Normal	Mean: 40.68 (2.06)	SD: 17.59 (1.46)	629.79
Exponential	Rate: 0.0246 (0.0029)	-	689.02
Postweaning period			
Log-normal	Mean: 3.45 (0.06)	SD: 0.47 (0.04)	513.98
Gamma	Shape: 4.03 (0.70)	Rate: 0.11 (0.02)	526.11
Logistic	Location: 32.06 (2.10)	Scale: 9.86 (1.07)	543.37
Normal	Mean: 35.74 (2.90)	SD: 22.86 (2.05)	568.01
Exponential	Rate: 0.0280 (0.0035)	-	569.47

AIC = Akaike Information Criterion

Table 3 Best fitting probability density functions to hair dehydroepiandrosterone sulphate distribution in postnatal and postweaning periods of calves

Function	Parameter 1 (± SE)	Parameter 2 (± SE)	AIC
Postnatal period			
Log-normal	Mean: 4.47 (0.05)	SD: 0.43 (0.04)	739.52
Gamma	Shape: 5.25 (0.84)	Rate: 0.05 (0.01)	747.45
Logistic	Location: 88.79 (4.54)	Scale: 23.11 (2.34)	762.19
Normal	Mean: 96.40 (5.67)	SD: 48.46 (4.01)	777.77
Exponential	Rate: 0.0104 (0.0012)	-	815.01
Postweaning period			
Log-normal	Mean: 3.62 (0.05)	SD: 0.39 (0.04)	512.79
Gamma	Shape: 6.43 (1.13)	Rate: 0.16 (0.03)	516.04
Logistic	Location: 38.35 (1.94)	Scale: 8.90 (0.95)	525.26
Normal	Mean: 40.24 (2.18)	SD: 17.14 (1.54)	532.32
Exponential	Rate: 0.0249 (0.0032)	-	584.16

AIC: Akaike Information Criterion

Table 4 Median [minimum, maximum] values of hair hormones in healthy and traumatized animals

Hair hormone	Clinically healthy calves		Traumatized calves	*p* value
	Postnatal period	Postweaning period		
Cortisol (pg/mg)	16.06 [1.38, 36.10]^{B}	4.74 [1.15, 17.50]^{A}	28.34 [21.87, 60.83]^{C}	< 0.001
DHEA (pg/mg)	40.96 [15.04, 115.56]^{a}	28.06 [12.02, 158.11]^{b}	35.13 [19.89, 57.91]^{ab}	0.026
DHEA-S (pg/mg)	86.06 [32.05,289.05]^{A}	37.02 [18.02, 104.27]^{B}	54.22 [25.91, 72.41]^{B}	< 0.001
Cortisol:DHEA *100	40.68 [3.27, 229.85]^{B}	17.11 [2.36, 57.39]^{A}	83.72 [51.82, 281.05]^{C}	< 0.001
Cortisol:DHEA-S *100	16.84 [1.58, 44.96]^{B}	12.25 [4.40, 40.02]^{A}	80.21 [36.39, 234.77]^{C}	< 0.001
DHEA:DHEA-S	0.43 [0.08, 1.58]^{A}	0.80 [0.42, 3.00]^{B}	0.71 [0.52, 1.07]^{B}	< 0.001

DHEA = dehydroepiandrosterone; DHEA-S = dehydroepiandrosterone sulphate

Values in the same row superscripted with different uppercase or lowercase letters are highly significantly ($p < 0.01$) or significantly ($p < 0.05$) different, respectively.

The highest ($p < 0.01$) hair cortisol concentration, cortisol:DHEA ratio and cortisol:DHEA-S ratio were in the TRA, while the lowest ($p < 0.01$) were in the PWP. The DHEA concentrations were higher in the POP than in the PWP ($p < 0.05$) and the TRA group showed similar DHEA concentrations compared to clinically healthy calves both in the POP and PWP ($p > 0.05$). The highest DHEA-S concentrations were in the POP ($p < 0.01$), with the TRA showing similar concentrations compared to the PWP group ($p > 0.05$). Conversely, the lowest DHEA:DHEA-S ratio was in the POP ($p < 0.01$), with the TRA showing similar concentrations in comparison to the PWP group ($p > 0.05$).

Discussion

The neonatal period is characterized by several environmental changes faced by newborn calves, with an endocrine setting that allows them to cope with the allostatic load of this particular period (Hammon et al., 2012). As described by Weary et al. (2008), several postnatal stressors (early separation from the mother, dietary change from the mother's milk to the bulk tank milk, introduction into new living area and modifications of social groups) can affect HPA-axis activity and consequently affect the development of the animal. It is also known that a calf that adapts badly to this period has a high chance of presenting lower productivity throughout its life; for example, the heifer stage is the weak link in many dairy farms, and if not well managed, it can cause serious losses, both in economic and animal welfare terms (Fantini, 2009; Bazeley et al., 2016). Despite a number of commercially available biosensors that quantify stress responses through parameters such as heart rate variability, rectal temperature and respiration rate, resting, laying and ruminating (Riaboff, 2020) the fruitfulness of such technological approaches has yet to be evaluated.

The "traditional" endocrine approach combined to a newer specimen as hair might be an interesting way for individual evaluations in calves and also on a large scale.

To date, several studies have been carried out on calves to investigate hair cortisol concentrations. At around age 60 d, Probo et al. (2021) found beef calves had lower hair cortisol concentrations than those in the current study on dairy calves, while at 150 days the cortisol values were common in both studies; Braun et al. (2019) at this time-point measured lower cortisol concentrations for non-regrowth samples taken at the slaughterhouse. The difference at age 60 d could be surely explained by the non-regrowth sample analyzed in the current study that also included the higher cortisol concentrations characteristic for the newborn calf (Maiero et al., 2005; Comin et al., 2008; González-de-la-Vara et al., 2011). This study was the first published report on the measurement of hair DHEA concentrations in calves, while the only study reporting hair DHEA-S concentrations in calves (Probo et al., 2021) was in accord with the concentrations observed in the current trial.

The analysis of the hair cortisol, DHEA, and DHEA-S concentrations from the hair samples collected at around age 60 d (at the end of the weaning) showed that all the animals had significantly higher hormone concentrations than those found at age 5 mth, excepting the hair cortisol concentrations measured in the seven calves that were stuck for a certain time in the feed fence (the TRA group). The first hair sample not only includes information linked to the extrauterine life, but also events that occurred during pregnancy and birth. In fact, this matrix is capable of cumulatively recording hormonal variations. Therefore, hair hormone concentration is assumed to be a retrospective marker of integrated hormone secretion over longer periods (Dettenborn et al., 2010; D'Anna-Hernandez et al., 2011; Pereg et al., 2011; Stalder and Kirschbaum, 2012). Several studies have shown that pregnancy plays a very important role in the future of the unborn animal (Funston et al., 2010; González-Recio et al., 2012; Noya et al., 2019) and that high cortisol levels have an important effect on the activity of many systems (Uetake et al., 2014; Strong et al., 2015) and fetal programming (Holt, 2002; Xiong and Zhang, 2013).

The reduction in hair cortisol concentrations at about age 5 mth compared to those measured at age 60 d demonstrated a reduction in the animal's allostatic load. This was not surprising, since in the postweaning rearing period, the number of environmental changes greatly reduced. Furthermore, it is conceivable that an increase in the calves' resilience did not result in an increase in DHEA and DHEA-S concentrations by itself but rather in a significant reduction in the cortisol:DHEA and cortisol:DHEA-S ratios, as already observed in piglets (Fels et al., 2019) and dairy cows (Peric et al., 2017).

The TRA group, which were stuck in the feeding fence for several hours at weaning time and that probably re-experienced this trauma every time they entered the feeding rack to feed, showed significantly higher cortisol concentrations compared to the animals sampled at age 5 mth and even to those sampled earlier. The cortisol:DHEA and cortisol:DHEA-S ratios of the TRA group were also significantly higher than those of the POP and PWP groups. This suggested that an acute stressor, such as trauma, can transform into a chronically repeated feeling that triggers a recurrent activation of the HPA axis. On the other hand, the TRA group demonstrated a similar average daily weight gain to the PWP group having, thus overcome the possible influence of the dysregulated HPA axis on the metabolic rate of the animals.

In conclusion, the simultaneous evaluation of cortisol, DHEA and DHEA-S in hair samples and their ratios may provide more information than an assessment based on each steroid alone, as it provides a more complete picture of the hypothalamic-pituitary-adrenal axis activity and functionality. Such assessments appear to be crucial to capitalizing on the genetic potential of livestock, to safeguard both animal welfare and their productive life. In addition, this may represent a preparatory phase in the development of predictive clustering trees (Nikoloski et al., 2019), which will aid machine learning tools and decision-making processes. Indeed, cortisol, DHEA and DHEA-S are steroid hormones related to the organism's ability to cope with environmental changes. Consequently, they affect the development of an animal. Evaluation of the hair cortisol, DHEA and DHEA-S can provide useful information about the ability of a calf to react to environmental changes; thus, facilitating the identification of calves with a higher allostatic load that may interfere with their ability to be resilient.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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