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Effect of experimental stress and cortisol release induced by ACTH administration on expression of key genes related to milk synthesis and apoptosis during mammary involution of Saanen goats

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Research Article

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Abstract

This research paper addresses the hypothesis that stress, induced by ACTH administration and cortisol release increases somatic cell count (SCC) in mammary secretion, and improves the effectiveness of dry off in goats. We report indicators of milk synthesis and mammary gland involution during dry off. Thirty Saanen goats were subjected to abrupt dry off and treatments: (1) ACTH administration (ACTH) or (2) placebo (Control) on days 1, 3, 6, 9, 12, 15, 30, and 60 of dry off. The expression of target genes in mammary tissue that are related to milk synthesis and cell survival such as insulin-like growth factor 1 receptor (IGF1R), phosphatidylinositol-3-kinase (PIK3CA), protein kinase B (AKT1) and mechanistic target of rapamycin (MTOR), casein (CSN2), lactalbumin (LALBA) and lactoferrin (LF) were evaluated, and plasma cortisol concentration, SCC, leucocyte count, and microbiological analyses in milk and mammary secretions were assessed. ACTH significantly downregulated the expression of IGF1R and upregulated the expression of PIK3CA in mammary tissue, increased lactoferrin concentration and SCC, and changed immune cell levels in mammary secretions compared to Control. Furthermore, ACTH administration increased the percentage of dry goats compared to the Control (73 *vs.* 46%, respectively). We conclude that the effect of stress *via* ACTH administration and cortisol release accelerated mammary involution during the early dry-off period.

Dry off accelerates apoptosis and involution of the mammary gland (Capuco and Akers, 1999; Caja *et al.*, 2006; Zhao *et al.*, 2019). Feed restriction may be used to decrease milk synthesis at the end of lactation in cows (Herve *et al.*, 2019; Vanacker *et al.*, 2020) and improve the efficiency of mammary involution after milking cessation (Ollier *et al.*, 2014). Dairy goats are usually managed in the same way, although they have rather persistent lactations, and some may continue to produce milk through until the next parturition if milking continues (Salama *et al.*, 2005; Caja *et al.*, 2006; Safayi *et al.*, 2010). Stress and glucocorticoids release have been associated with increased apoptosis and decreased milk production in cows and sheep (Caroprese *et al.*, 2010; Ollier *et al.*, 2016; Ponchon *et al.*, 2017), however, whether this might also affect apoptosis and involution during the dry period of goats remains unclear.

Feed restriction and then abrupt cessation of milking are considered stressful management (Odensten *et al.*, 2007; Zobel *et al.*, 2013). Some authors have also associated milk accumulation during the dry period to stress, inflammation of the mammary gland and a higher risk of mastitis (Bertulat *et al.*, 2013; Mehdid *et al.*, 2019; Zhao *et al.*, 2019). Other studies have reported increased infiltration of immune cells and an increased rate of apoptosis in mammary tissue within a few hours or days after the start of the dry off (Boutinaud *et al.*, 2016; Lanctôt *et al.*, 2017; Singh *et al.*, 2017). Moreover, milking cessation is marked by decreased casein and lactalbumin synthesis and an increase of lactoferrin synthesis in mammary tissue (Singh *et al.*, 2017). In addition, the reduction of alveolar tissue can be observed within weeks after the start of dry off (Capuco *et al.*, 2001; Singh *et al.*, 2017).

The dry off coincides with the estrogen changes that characterize the acceleration of mammary involution and the end of pregnancy. These changes induce the renewal of epithelial cells, resulting in a lobe-alveolar structure that ensures copious milk synthesis during the next lactation (Safayi *et al.*, 2010; Zhao *et al.*, 2019). In this context, the analyses of the expression of casein (CSN2), lactalbumin (LALBA), and lactoferrin (LF) genes related to capacity synthesis and immunomodulation in mammary tissue during dry off are essential to understand the involution (Singh *et al.*, 2005, 2017). Likewise, the expression of insulin-like growth

factor 1 receptor (IGF1R) genes and phosphatidylinositol-3-kinase (PIK3CA), protein kinase B (AKT1), mechanistic target of rapamycin (MTOR), all of which are related to apoptosis control in mammary epithelial cell line (Burgos *et al*., 2010; Li *et al*., 2017), can contribute to understanding the effects of stress and cortisol release during dry off.

We hypothesized that stress induced experimentally by ACTH administration and cortisol release would increase SCC in mammary secretions, and improve the effectiveness of dry off in Saanen goats. Our objective was to evaluate the effect of ACTH administration on the expression of genes related to milk synthesis (LALBA, CSN2) and antimicrobial action (LF), genes related to cell survival and apoptosis control (IGF1R, PIK3CA, AKT1, MTOR) in mammary tissue, and its relationship with lactoferrin concentration, SCC and leukocyte count in mammary secretions of Saanen goats during dry off.

Materials and methods

Experimental procedures were approved by the Animal Ethics Committee (protocol 3709280316) of the Faculty of Animal Science and Food Engineering in accordance with Brazilian federal law.

Housing, food, and management

The Saanen goats remained in collective pens with free access to feed and water troughs and mineral salt. The goats were fed with 50% roughage (containing corn silage) and 50% concentrate (corn and soybean meal, soybean oil, limestone and mineral and vitamin mix). During lactation, the diet was adjusted every 2 weeks, considering the live weight and milk production to ensure leftovers of 15% of the total diet provided (NRC, 2007). Throughout lactation, the experimental goats were subjected to one daily milking (7 am). In the last milking, all goats received an intramammary antibiotic (Ouro Fino, Cravinhos, SP, Brazil) as a preventive measure against mammary gland infections. During the last 3 d of lactation (on day 247 ± 5 of lactation) and after abrupt cessation of milking on day 250, all the goats began receiving a dry off goat diet. This diet contained total restriction of corn silage and concentrate, and the goats received Tifton 85 hay *ad libitum* for a period of 15 d. Afterwards, the animals received a dry goat diet containing corn silage and concentrate (NRC, 2007). The experimental procedures were performed in 12 h of daylight and 12 h of darkness.

Organization of the experiment

No goats were pregnant during our experiment because the objective was to study the effect of stress, *via* ACTH administration and cortisol release on mammary involution during dry off without the influence of pregnancy. On day 250 of lactation (experiment day 0), the 30 goats (67.1 ± 2.5 kg of BW, 3.2 ± 0.1 of body score, 2.2 ± 0.5 lactations) were subjected to abrupt dry off and were blocked (according to parity number, number of kids born, total milk yield in current lactation, body weight and body condition score) in two treatments (1) challenge with ACTH (intravenous administration of 0.6 IU/kg per BW per animal) and (2) Control (saline administration).

The ACTH and Control treatments were administered by intravenous injections at 7 am on days 1, 3, 6, 9, 12, 15, 30, and 60 of dry off. The ACTH dose used causes a cortisol peak at 60 min and a return to baseline cortisol concentrations at 240 min after ACTH administration (Fulkerson and Jamieson, 1982; Bomfim *et al*., 2018).

Sampling and analyses

Milk yield (kg/d) was recorded during the last 15 d of lactation. In order, blood samples, milk samples and mammary secretions were collected at 8 am (1 h after ACTH or Control administration) before (day −1, on day 249 of lactation) and after milking cessation of the experimental goats, on days 1, 3, 6, 9, 12, 15, 30, and 60 of the dry period. Milk and mammary secretion samples were taken from both teats manually and mixed about 50 ml to determine the composition, microbiological content, SCC, leukocyte count and lactoferrin concentration. Blood samples were taken by a jugular venepuncture. Cortisol in plasma was determined by an EIA kit (Monobind, Lake Forest, CA, USA), the intra- and inter-assay coefficients of variation were less than 4.3 and 6.8%, respectively. The lactoferrin concentration in milk and mammary secretions was determined by an EIA kit (Bethyl Laboratories, Montgomery, Texas, USA). The fat, protein and lactose levels were determined by an ultrasound equipment (Scope Electric®, Razgrad, Bulgaria). The SCC and leukocyte count in milk and mammary secretions were performed using the direct method (Dulin *et al*., 1982; Gonzalo *et al*., 2004).

Experimental goats were considered dry when the volume of mammary secretion collected was less than 50 ml (Fleet *et al*., 1975). Photographs of the udders were assessed on days 1, 3, 6, 9, 12, 15, 30, and 60 of the dry off to visually confirm the involution of the mammary gland (Fowler *et al*., 1991). The involution was considered total when the udder had a morphology similar to those observed in non-lactating and non-pregnant goats.

Gene expression

On days 1, 15, 30, and 60 after the start of dry off, biopsies were performed on the mammary gland of eight goats (four ACTH goats and four Control goats) distributed to milk yield and representative of other experimental goats. Biopsies were also performed 60 min after ACTH or Control administration, as previously described (Bomfim *et al*., 2018; Hooper *et al*., 2020). Briefly, the disposable biopsy needle was inserted into the incision to a depth of approximately 3.0 cm, and the equipment was triggered to cut and collect a sample of approximately 2.0 × 0.5 cm of mammary tissue. The relative levels of gene expression of IGF1R, PIK3CA, AKT1, MTOR, CSN2, LALBA, and LF proteins were determined through real-time quantitative PCR using the StepOne Real-Time PCR System (Invitrogen) as well as the primers described in online Supplementary Table S1. The total RNA of mammary tissue (80 to 100 mg) was extracted and purified using a PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). The material was treated with RNase-free DNase (Promega, Madison, WI, USA) to prevent genomic DNA contamination. The RNA concentrations were determined using Qubit 2.0 Fluorometric Quantification (Thermo Fisher Scientific, Waltham, MA). The quality of each RNA sample was evaluated by the optical density at the 260 and 280 nm absorbance waves and the 260/280 absorption ratio was approximately 2. The integrity of the RNA was analyzed on denaturing agarose gel. The relative expression of the gene was quantified using the Livak method ($\Delta\Delta Ct = 2^{-\Delta\Delta Ct}$) (Livak *et al*., 2001) to compare the expression level of the target genes with the reference (GAPDH). For each

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primer set, the PCR efficiency was close to 100%, and the specificity of the primer pairs was confirmed through analysis of the melting curve and appropriate size, using 1.5% agarose gel electrophoresis.

Statistical analysis

Data normality was confirmed using the Shapiro–Wilk test. The effects of treatment (ACTH or Control) on gene expression, cortisol in plasma, lactoferrin concentration, SCC, leukocyte count, and microbiological analysis of mammary secretions during the dry period was analyzed through ANOVA using the MIXED procedure of SAS 9.4, which separated the effects of treatment, day, and goats as causes of variation in the model; treatment and day was considered a fixed effect and goats were considered random. Several covariance matrices were tested, and the one that had the best structure according to Bayesian information and criteria was selected. When there was a significant effect, the means were compared using Fisher's test with a significance level of $P \leq 0.05$. Data are presented as mean ± standard error.

Results

As expected, feed restriction significantly decreased the milk yield of the experimental goats (online Supplementary Fig. S1). The cortisol concentration of goats submitted to ACTH was significantly higher than that measured for goats Control (online Supplementary Fig. S2). In addition, the increase in cortisol concentration in Control goats during the dry off confirmed that this practice was stressful for goats (online Supplementary Fig. S2). The results obtained demonstrated that the stress caused *via* ACTH administration causes a significant increase in the percentage of dry goats at days 6, 15, 30 and 60 compared to the Control (Table 1). At the same time, dry goats from both experimental groups visually presented total udder involution, showing a similar morphology of non-lactating and non-pregnant goats (online Supplementary Fig. S3).

The highest cortisol in ACTH goats significantly increased the concentration of lactoferrin, milk density, SCC and percentage of eosinophils and basophils, but decreased the concentration of lymphocytes compared with the mammary secretions of the Control goats (Table 2; online Supplementary Figs S4 and S5). Comparing the treatment effect on gene expression during the entire dry period (calculated as gene expression on day 60 *vs.* day 1 of dry period for ACTH or Control treatments), ACTH goats significantly downregulated the expression of IGF-1R and upregulated the expression of PIK3CA in mammary tissue when compared with goats submitted to the Control (Fig. 1). Comparing the effect of day biopsy, the expression of AKT1, LF, LALBA, and CSN2 genes on day 1 was significantly higher when compared to day 60 of dry off (Fig.2).

Table 1. Effect of ACTH or Control treatment on percentage of dry goats during the dry period of Saanen goats

	Treatment		
Percentage of dry goats/days[1]	ACTH	Control	*P*-value[2]
1 (%)	0	0	–
3 (%)	0	0	–
6 (%)	6.6[a]	0[b]	0.01
9 (%)	6.6[a]	6.6[a]	0.99
12 (%)	20[a]	13[a]	0.09
15 (%)	33[a]	20[b]	<0.01
30 (%)	46[a]	33[b]	<0.01
60 (%)	73[a]	46[b]	<0.01

[1]Experimental goats were considered dry when the volume of mammary secretion collected was less than 50 ml.
[2]The percentages were compared using a χ^2 test.
[a,b]Percentage within a row with different superscripts differ ($P \leq 0.05$).

Discussion

Feed restriction significantly reduced milk yield before the abrupt cessation of milking as the lower availability of nutrients decreased the milk synthesis by the mammary gland (Ollier *et al.*, 2016; Herve *et al.*, 2019; Vanacker *et al.*, 2020). ACTH administration increased the percentage of dry goats compared to the Control (73 *vs.* 46%). On the other hand, 56% of goats submitted to Control had persistent lactation just 60 d after the start of dry off. These results confirm that typical dry off management was ineffective, and many goats have a considerable volume of mammary secretion in the mammary gland. Some authors have suggested that feed restriction causes hunger and interruption of milking that causes discomfort and even pain, stressors that increase cortisol concentration during dry off (Bertulat *et al.*, 2013; Zobel *et al.*, 2015). In addition, ACTH administration significantly increased the concentration of cortisol and the percentage of dry goats, improving the efficiency of dry off when compared to Control goats.

Other authors have also reported that abrupt cessation of milking causes milk accumulation, decreases the synthesis of the main constituents of milk and increases the permeability of alveoli, changing the composition of mammary secretions at the start of dry off (Singh *et al.*, 2016, 2017). In our study, the higher density and lactoferrin concentration in mammary secretions of goats submitted to ACTH confirms its effect in the mammary gland. The large percentage variability of the main constituents in mammary secretions observed in our study suggested that the dry off effect on goats with persistent lactations is quite heterogeneous. In contrast to that reported by other authors (Fowler *et al.*, 1991), our results also demonstrate that dry goats showed an udder morphology similar to that observed in non-pregnant and non-lactating goats. Indeed, goats with persistent lactation during the dry off process also showed visual changes in udder morphology, even when they maintained a considerable volume of secretion in the udder at the end of our study. In our study, goats were not pregnant, and we cannot associate lactation persistency to hormonal variations that characterize the end of pregnancy and accelerate mammary gland renewal, as reported by other authors (Salama *et al.*, 2005; Safayi *et al.*, 2010).

Furthermore, our results demonstrate that ACTH administration significantly downregulated the expression of IGF1R and upregulated the expression of PIK3CA in mammary tissue compared to the Control. Consequently, as we initially postulated, the increased cortisol levels in ACTH goats changed the expression of genes IGF1R and PIK3CA in the IGF1R/PIK3CA/AKT1/MTOR pathway. However, ACTH administration decreased the expression of IGF1R and increased the expression of PIK3CA, both associated with an anti-apoptotic role and cell survival (Burgos *et al.*, 2010; Zhang *et al.*, 2018). The ACTH effect

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Table 2. Effect of ACTH or Control treatment (T), sampling day (D), and their interaction (T*D) on composition, somatic cell count, leukocyte count, and microbiological analysis in the mammary secretion of Saanen goats during the dry period. Data are presented as the mean ± standard error of the mean.

	Treatment					
	ACTH[1]	Control[1]			*P*-value	
Item	(*n* = 15)	(*n* = 15)	SEM	T	D	T*D
Lactoferrin (ng/ml)	2.65[a]	1.81[b]	0.37	0.03	0.11	0.05
Lactose (%)	4.42	4.51	0.21	0.86	0.19	0.59
Protein (%)	3.24	3.31	0.14	0.86	0.20	0.58
Fat (%)	3.37	3.44	0.43	0.98	<0.01	0.67
Total solids (%)	11.83	10.04	0.35	0.77	0.15	0.60
Density (g/ml)	1.030[a]	1.027[b]	0.01	0.01	0.62	0.56
Dry extract (%)	8.24	8.32	0.31	0.80	0.35	0.77
Somatic cell count (cells/ml)[2]	1.912[a]	1.474[b]	0.22	<0.01	<0.01	0.25
Eosinophils (%)	6.48[a]	5.44[b]	0.44	0.01	<0.01	0.15
Basophils (%)	2.76[a]	2.43[b]	0.20	0.04	0.05	0.02
Neutrophils (%)	54.78	55.16	0.99	0.78	0.01	0.59
Lymphocytes (%)	31.52	32.37	0.95	0.37	<0.01	<0.01
Macrophages (%)	4.46	4.56	0.29	0.75	<0.01	0.36
Total count of bacteria (CFU/ml)[3]	3.45	1.47	1.99	0.32	0.17	0.39
Enterobacteriaceae (CFU/ml)[4]	–	–	–	–	–	–
Staphylococcus sp. (CFU/ml)[3]	3.42	1.78	1.99	0.41	<0.01	0.95

[1]Four goats from ACTH and eight goats from the Control group showed a considerable volume of mammary secretion and were no considered dry goats.
[2]Values × 10^3/ml.
[3]CFU: colony-forming units, values × 10^3/ml.
[4]The count was not high enough to perform statistical analysis.
[a,b]Means within a row with different superscripts differ ($P \leq 0.05$).

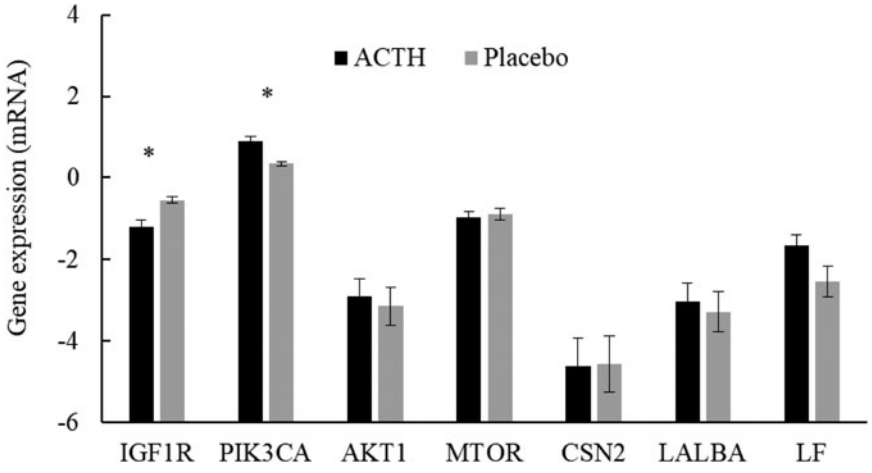

Fig. 1. Downregulation and upregulation of target genes in mammary gland for goats subjected to ACTH and Control during the entire dry period. Gene expression is reported as fold change ($2^{-\Delta\Delta Ct}$) relative to Control. Gene expression during the entire period was presented as gene expression on day 60 *vs.* day 1 of the dry period for ACTH and Control treatments. The asterisk (*) indicates significant differences ($P \leq 0.05$) between ACTH (black bars: gene expression on day 60 *vs.* day 1) and Control (gray bars: gene expression on day 60 *vs.* day 1) treatments.

was higher on the expression of IGF1R than on the expression of PIK3CA, suggesting that this misbalance accelerated mammary involution in goats subjected to ACTH administration. The lower expression of the IGF1R gene in mammary tissue of ACTH goats was also related to an increase in SCC in mammary secretion and a higher percentage of dry goats when compared to Control goats (73.3 vs. 46.7%, respectively). Moreover, ACTH also increased the lactoferrin concentration in mammary secretions when compared to Control. Other authors have reported that in the absence of sub-clinical or clinical mastitis, the increase in SCC is a consequence of the increase in epithelial cells exfoliated in milk (Boutinaud *et al.*, 2016; Herve *et al.*, 2016). In our study, there was no effect of ACTH on microbiological status or intramammary infections, so we can argue that there was a higher exfoliation of the mammary epithelial cells during the mammary involution of the goats subjected to ACTH administration. Although the total number of mammary epithelial cells decreases during lactation of the goats (Knight and Peaker, 1984; Safayi

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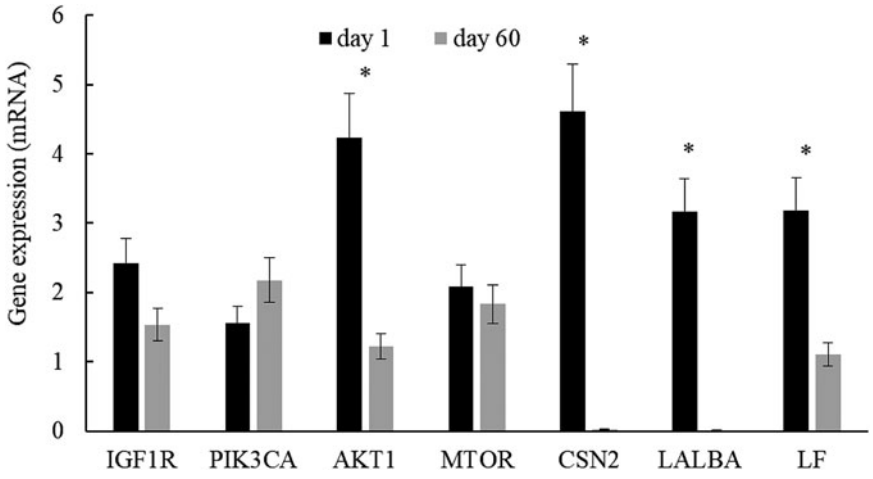

Fig. 2. Gene expression in mammary tissue of experimental goats on days 1 and 60 of dry off. Gene expression is reported as fold change ($2^{-\Delta\Delta Ct}$) relative to Control. The asterisk (*) indicates significant differences ($P \leq 0.05$) between day 1 (black bars) and 60 (gray bars) of dry off.

et al., 2010), some authors have argued that the goats have more persistent lactations because their epithelial cells that survive maintain their high capacity for milk synthesis (Knight and Peaker, 1984; Wilde *et al.*, 1997; Safayi *et al.*, 2010). These last aspects can partially explain why 54% of goats submitted to the Control and 27% of goats submitted to ACTH had a considerable secretion volume in their mammary gland 60 d after the start of dry off. As previous studies concerning cortisol supplementation in epithelial mammary cells of goats demonstrated increase apoptosis (Bomfim *et al.*, 2018), further studies with ACTH administration are necessary to determine its effects on apoptosis of epithelial cells and mammary involution during the dry period.

In our study, ACTH goats presented significantly higher basophil and eosinophil percentages and lower lymphocyte percentage in mammary secretions than Control goats. This changeover can be attributed to ACTH and cortisol because both are immunosuppressants (Sordillo *et al.*, 1997; Gonçalves *et al.*, 2017; Mehdid *et al.*, 2019). In contrast, the neutrophil percentage, the most abundant immune cells during the inflammatory process of mammary tissue in ruminants (Sordillo *et al.*, 1997; Gonçalves *et al.*, 2017), was not influenced by ACTH. For this reason, we argue that the physiological ACTH dose used had a limited effect on the immune response. Furthermore, the ACTH goats had an increased lactoferrin concentration in mammary secretions compared to Control, which might partially explain why our goats did not present clinical mastitis during dry off. Lactoferrin is bacteriostatic and can prevent growth of bacteria such as staphylococci and coliforms, which have iron requirements (Sordillo *et al.*, 1997). In addition, administering intramammary antibiotics for all experimental goats was effective, since no goats presented clinical mastitis during dry off. On the other hand, the decrease of lymphocytes in mammary secretions, which actively participate in the immune response in the mammary gland, is a concern and may hinder the use of higher doses of ACTH than that used in our study.

In conclusion, our study shows that ACTH administration significantly downregulated the expression of IGF1R and upregulated the expression of PIK3CA in mammary tissue and increased the lactoferrin concentration and SCC in mammary secretion compared to the Control. ACTH administration significantly increased the percentage of dry goats compared to the Control (73 *vs.* 46%), therefore, we can conclude that stress induced experimentally by ACTH administration and cortisol release accelerated mammary involution in ACTH goats during the early dry-off.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029922000735.

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