*Bulgarian Journal of Agricultural Science, 25 (No 3) 2019, 564–569*

# Discrepancy between plasma cortisol level and neutrophil-to-lymphocyte ratio in sheep during shearing

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## Abstract

Moneva, P., Yanchev, I., Dyavolova, M., & Gudev, D. (2019). Discrepancy between plasma cortisol level and neutrophil-to-lymphocyte ratio in sheep during shearing. *Bulgarian Journal of Agricultural Science, 25*(3)*,* 564–569

The object of the present study was to investigate the link between adrenal and leukocyte responses to shearing stress in sheep. The primary parameters determined in this study were cortisol dynamics and neutrophil-to-lymphocyte (N/L) ratio in lactating non-pregnant ewes from the Bulgarian synthetic dairy population (n = 20) and non-pregnant ewes from the Ile de France (meat type) breed (n=20) during shearing. The ewes were shorn at the beginning of June. Blood samples were taken before shearing, immediately after shearing and at 3 h following the onset of shearing.

Ile de France ewes were divided into two subgroups based on their reactivity to shearing as assessed by cortisol level immediately after shearing. Group I comprised ewes that had high cortisol level (more stress-reactive) and group II comprised ewes that had low plasma cortisol level immediately after shearing (less stress-reactive). Cortisol level in the ewes of Bulgarian synthetic dairy population (BSDP) was higher immediately after shearing ($P < 0.001$) as compared to Ile de France ewes. Plasma cortisol level in the ewes of Bulgarian synthetic dairy population (BSDP) declined significantly at 3 h after the onset of shearing while at that time, cortisol levels in the other group tended to decline but did not reach level of significance. There was significantly higher N/L ratio in BSDP ewes as compared to Ile de France ewes immediately after shearing. Neutrophil-to-lymphocyte ratio increased significantly in both breeds of sheep at 3 h following the onset of shearing. Plasma cortisol levels in the more reactive Ile de France ewes were higher across all measurements as compared to less reactive ewes while N/L ratio immediately after shearing and at 3 h since the onset of shearing was higher in less stress-reactive ewes. These results suggest that the magnitude of cortisol response to acute stress is not matched by a corresponding increase in N/L ratio. The results are interpreted to mean that total plasma cortisol level plays a lesser role than free cortisol in N/L trafficking and that neutrophil elastase-induced cleavage of corticosteroid-binding globulin regulates the amount of free cortisol.

*Keywords:* cortisol; neutrophil-to-lymphocyte ratio; shearing; stress; reactivity; breed; sheep

## Introduction

Much of the early literature points to a close link between leukocyte profiles and glucocorticoid levels. Glucocorticoid hormones are generally accepted to increase the number and percentage of neutrophils, while decreasing the number and percentage of lymphocytes (Davis et al., 2008). Changes in circulating leukocytes in response to various stressors were documented in early stress research. Decreases in lymphocytes and eosinophils and increases in neutrophils occur in pigs fed with increasing concentrations of dietary cortisol (Widowski et al., 1989), and chickens exposed to multiple concurrent stressors (McFarlane and Curtis, 1989). The mechanism underlying the effect of stress hormones on leukocyte distribution has been reviewed by Brenner et al. (1998). Heterophil and lymphocyte numbers change more slowly in response to stress than does corticosterone (Maxwell, 1993). Recently, it has been shown that short-time

(minutes) of psychological stress, unlike chronic (longer time of mental stress), may improve immune performance. Also, it has been shown that minutes of mental stress induces the production of proinflammatory cytokines (a Th1 like response) via a mild and transient increase in cathecolamines and cortisol (Matalka, 2003). Neutrophils are the major population of leukocytes. They are one of the first cell types at sites of inflammation. Altstaedt et al. (1996) suggested that IL-8 is the only cytokine neutrophils are able to release at significant levels and that neutrophil-directed immune regulation is driven by IL-8. In contrast, other investigation has shown that the rapid recruitment of neutrophils to sites of inflammation may be an important source of proinflammatory mediators such as IL-1 (Basran et al., 2013). Human and murine neutrophils have been shown to express proinflammatory/anti-inflammatory cytokines, chemokines, immunomodulatory cytokines and angiogenic/fibrogenic factors (Tecchio et al., 2014). Overall, the literature demonstrates that stress hormones stimulate leukocyte trafficking to sites of inflammation and exert stimulatory effect on immune cells during exposure to acute stress. Widowski et al. (1989) reported difference in N/L ratio between pig trials even when dietary cortisol levels were similar. These data suggest that factors, other than stress hormones, are implicated in the control of N/L ratio.

The object of the present study was to investigate inter- and intra-breed dynamics of cortisol and N/L in response to shearing in sheep and the association between plasma cortisol level and N/L ratio.

## Materials and Methods

Twenty non-pregnant, lactating ewes from the Bulgarian dairy synthetic population (BDSP) and 20 non lactating ewes in the first two months of pregnancy from Ile de France breed were included in the present experiment. Ile de France ewes were artificially inseminated following induced ovulation in the beginning of April. All ewes were randomly selected from the respective sheep breed herds at the Institute of Animal Science, Kostinbrod. The average age of the ewes was 3 years. During winter months the ewes were kept in barns with deep straw litter and were fed meadow hay, corn silage and concentrate according to their nutritional requirements. Hay was fed in racks and concentrate was offered in grain feeders. Sheep had permanent access to clean water. They had year round access to a fenced-in yard situated next to the barn. Ile de France ewes were allocated into two subgroups according to their stress-reactivity (assessed as plasma cortisol level immediately after shearing). Group I comprised individuals with high level of cortisol (more stress-reactive, n = 10) and group II comprised individuals with low level of cortisol (less stress reactive, n=10). The ewes were shorn at the beginning of June and sheep shearing started at 10 am. Each sheep was shorn within 10 min. Minimum and maximum temperatures on that day were 13 and 25°C respectively. Blood samples were taken before shearing, immediately after shearing (at 10 min following the start of shearing) and at 3 h following the onset of shearing. All samples were taken via jugular venipuncture within 3 min in order to minimize the effect of stress caused by the handling. Differential white blood cell count was performed. All leukocyte subpopulations were counted microscopically in smears made after staining with Giemsa-Romanovsky. In the current study we examined neutrophil-to-lymphocyte ratio as a simple parameter of systemic inflammation and stress. The results of one factor analysis are expressed as means ± S.E.M. and were analyzed by ANOVA.

## Results and Discussion

Basal plasma cortisol levels between the breeds were similar (Fig. 1). However, basal neutrophil-to-lymphocyte (N/L) ratio was significantly higher in BSDP sheep in comparison with Ile de France sheep (Fig. 2). It is well documented that epinephrine, norepinephrine and corticosteroids have a major role in neutrophil mobilization and trafficking (Dhabhar et al., 2012). Therefore, the observed difference in basal N/L ratio between the breeds despite the similar cortisol levels suggests that N/L ratio was influenced by a factor or factors other than stress hormones. Corticosteroid-binding globulin appears to be the major factor that may influence plasma cortisol level and thus glucocorticoids action in tissue. It has been reported that the gene encoding CBG (called SerpinA6) is strongly associated with cortisol levels. Also, studies conducted in different pig lines confirmed the genetic association between SerpinA6, CBG locus and basal cortisol levels in pig (Moisan & Castanon, 2016). Furthermore, Ousova et al. (2004) reported two times higher basal cortisol and three times higher CBG capacity in Meishan compared with Large White breeds. It is widely accepted that CBG actively deliver free glucocorticoids to inflamed tissue due to the action of elastase released by neutrophils. Corticosteroid-binding globulin is cleaved by neutrophil elastase, thereby reducing markedly glucocorticoids binding and enabling massive local delivery of free glucocorticoids which are biologically active (Hammond et al., 1990; Moisan & Castanon, 2016). Consequently, increased basal neutrophil count in BSDP sheep compared to Ile de France sheep could supply more elastase and therefore freer cortisol despite the similar levels of basal plasma cortisol levels in both breeds.

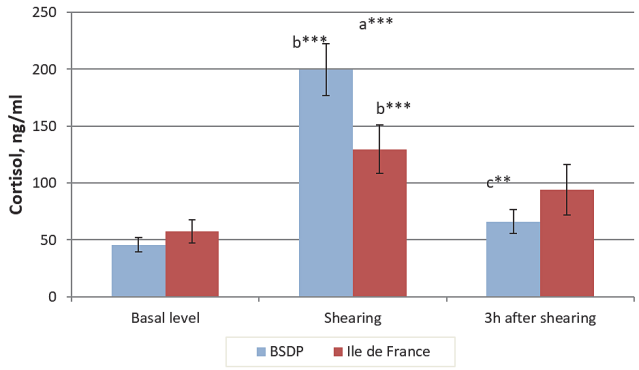


**Fig. 1. Effect of shearing on plasma cortisol levels in two breads of sheep**

a – significantly different among the breeds, b – significantly different versus respective baseline level, c – significantly different versus shearing; * p < 0.05, **p < 0.01, ***p < 0.001

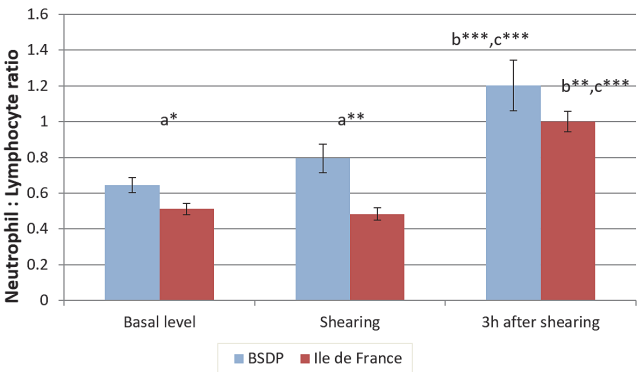


**Fig. 2. Effect of shearing on neutrophil-to-lymphocyte ratio in two breeds of sheep**

a – significantly different among the breeds, b – significantly different versus respective baseline level, c – significantly different versus shearing; * p < 0.05, **p < 0.01, ***p < 0.001

There was significant difference between the studied breeds in their sensitivity to shearing stress (Fig. 1). Plasma cortisol level immediately after shearing was higher in BSDP sheep compared to Ile de France sheep. Cortisol level immediately after shearing was assumed to reflect the level of stress-reactivity since each sheep was shorn within 10 min and, peak cortisol level in response to acute stress was reported to be at 60 min following the start of the stress stimulus (Verbeek et al., 2012). Neutrophil-to-lymphocyte ratio immediately after shearing increased slightly in BSDP sheep (P>0.05) but remained unchanged in Ile de France sheep which led to more pronounced interbreed difference (P < 0.01). The higher cortisol level in BSDP sheep immediately after shearing was probably due to higher stress-sensitivity of the breed compared to Ile de France breed. Mice with a genetic predisposition for high reactivity were reported to have higher basal and stress-induced levels of total corticosterone, CBG and free corticosterone compared with the mice having intermediate or low reactivity (Mattos et al., 2013). It has only recently been appreciated that a psychological or systemic stressor in the absence of overt tissue damage can also trigger systemic and central nervous system sterile inflammation (Fleshner et al., 2017). According to Dhabhar and McEwen (1999) a hormonal alarm signal released by the brain on detecting a stressor may prepare the immune system for potential challenges.

Plasma cortisol level in BSDP sheep declined sharply at 3h after the shearing whereas in Ile de France sheep cortisol level declined insignificantly at that time. The observed reduction in plasma cortisol levels in BSDP sheep coincided with a significant neutrophil-to-lymphocyte increase at that time (Fig. 2). We assume that cortisol decline in BSDP sheep at 3h following shearing was related to the higher level of both total and free cortisol immediately after shearing. This assumption is consistent with the reported higher CBG, total and free cortisol levels following 15 min restraint and 6 min swimming stress in mice selected for extremes in stress reactivity (Mattos et al., 2013). It is widely recognized that acute or short-term stress induces a rapid and significant distribution of immune cells among different body compartments and that adrenal stress hormones are major mediators of this leukocyte distribution. Furthermore, acute stress neutrophilia was suggested to favor immunoenhancement by mobilizing neutrophils to the blood stream and making them available for recruitment and activation at sites of inflammation (Dhabhar, 2002a; Dhabhar, 2002b). Also, neutrophils were reported to release cytokines and orchestrate the immune/inflammatory response by establishing bidirectional interactions with dendritic cells, monocytes, macrophages and T cells (Cerutti et al., 2013; Mócsai, 2013). Certain proinflammatory cytokines have been demonstrated to activate the stress system in vivo (Besedovsky et al., 1986; Chrousos, 1995; Kovacs et al., 1995; Akita et al., 1996).

It is generally thought that proinflammatory cytokines stimulate the release of stress hormones which in their turn prevent immune responses from becoming over-activated or autoimmune (Silverman et al., 2005). Therefore, it may be assumed that the higher cortisol level in BSDP sheep immediately after shearing was due to neutrophil orchestrated release of inflammatory cytokines which stimulated the stress axis. Besides, in addition to neutrophils-modulated pro-inflammatory cytokines, increased neutrophil count in BSDP sheep compared to Ile de France sheep may serve to increase

neutrophil elastase secretion, thereby reducing markedly corticosteroid-binding globulin and enabling massive delivery of free glucocorticoids at site of inflammation (Pemberton et al., 1988; Hammond et al., 1990; Moisan & Castanon, 2016). Consequently, increased N/L ratio at 3 h following shearing in both breeds could be considered as a delayed neutrophil response to increased cortisol level immediately after shearing. This view is consistent with the proposed specific role of epinephrine, norepinephrine and glucocorticoids in mediating mobilization and trafficking of leukocyte subpopulations (Dhabhar et al., 2012). Furthermore, it is widely recognized that unlike the hormonal response to stress, the initial leukocyte response begins over a time span of ours, depending on the species (Davis et al., 2008). The observed cortisol decrease in BSDP sheep at 3h after shearing could be due to higher total and free cortisol immediately after shearing since glucocorticoids are known to serve a vital function in negative feedback inhibition of their own secretion by diverse mechanisms, including fast, non-genomic feedback at the level of paraventricular nucleus (Herman et al., 2012). Also, increased number of neutrophils at that time could contribute to an increased supply of elastase that can cleave corticosteroid-binding globulin and promote the release of free glucocorticoids.

Basal neutrophil-to-lymphocyte ratio in the more stress-reactive sheep of Ile de France breed did not differ significantly from that in the less stress-reactive sheep (Fig. 4) irrespective of the significantly higher cortisol level at that time compared to cortisol level in the less stress-reactive sheep (Fig. 3). These data suggest a possible similarity of the basal free cortisol levels in both more stress-reactive and less stress-reactive sheep. Corticosteroid-binding globulin maximal capacity was reported to be 3 (1.7 to 5.2) times more than plasma cortisol levels in seven species including sheep. Also, it was found that 68% of CBG remains in a cortisol-free state under physiological conditions (Gayrard et al., 1996). Therefore, it could be assumed that under normal physiological conditions plasma levels of free cortisol in both groups is maintained within a similar range irrespective of total plasma cortisol concentration. Our assumption is consistent with the perception that CBG functions in maintaining a dynamic equilibrium between CBG-bound and unbound cortisol (Mattos et al., 2013).

Neutrophil-to-lymphocyte ratio in the more stress-reactive sheep (Fig. 4) immediately after shearing was lower than in the less stress-reactive sheep despite the higher cortisol level (Fig. 3) in the stress-reactive sheep at that time. In our previous investigations (in press) we found that the increase of N/L ratio starts at least 70 min, following exposure of sheep to acute stress and peaks at 150-180 min, after the onset of stress exposure. Therefore, it could be assumed that the observed N/L ratio immediately after shearing was due to catecholamines rather than to cortisol. This assumption is consistent with the hypothesis of Dhabhar at al. (2012) that norepinephrine and epinephrine mobilize immune cells into the blood stream while epinephrine and corticosterone induce trafficking out of the blood (Dhabhar et al., 2012).

Neutrophil-to-lymphocyte ratio increased sharply in both groups of sheep at 3 h following shearing. However the rate of increase was higher in the less stress-reactive

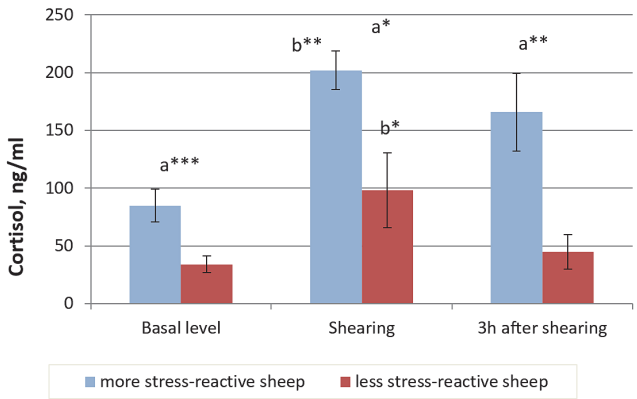


**Fig. 3. Effect of shearing on plasma cortisol levels in more stress-reactive and less stress-reactive sheep of Ile de France breed**

a – significantly different among the groups, b – significantly different versus respective baseline level; * p < 0.05, **p < 0.01, ***p < 0.001

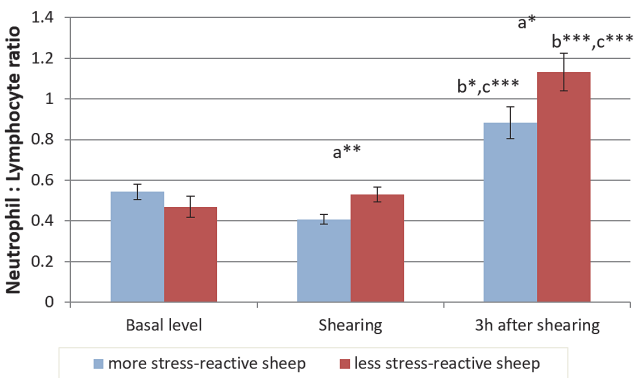


**Fig. 4. Effect of shearing on neutrophil-to-lymphocyte ratio in more stress reactive and less stress-reactive sheep of Ile de France breed**

a – significantly different among the groups, b – significantly different versus respective baseline level, c – significantly different versus respective shearing level; * p < 0.05, **p < 0.01, ***p < 0.001

sheep. These results suggest that higher cortisol level in the high-reactive sheep was buffered by a concomitant increase in CBG capacity thus resulting in low plasma free cortisol and consequently, less pronounced increase in N/L ratio. This suggestion is not consistent with the reported higher free cortisol in high-reactive mice despite higher plasma CBG (Mattos et al., 2013). The authors associated this phenomenon with increased corticosterone response to stressors that overloaded CBG buffering function. In contrast, Aberle et al. (1976) found significant interbreed variation of CBG binding capacity in pigs that was not related to their stress-susceptibility status. In our opinion, the higher N/L ratio in the less stress-reactive sheep at 3h after shearing was due to increased level of free cortisol caused by increased level of neutrophil elastase which may cause cleavage of CBG. It is known that the cleavage of CBG trigger structural rearrangements and reduced steroid binding affinity allowing release of free cortisol (Gardill et al., 2012). Our view is further supported by the reported inverse relationship between human plasma CBG and metabolic clearance rate of cortisol (Siiteri et al., 1982). Consequently, it could be assumed that lower cortisol level in the less stress-reactive sheep was due to a high clearance rate of cortisol. The lower cortisol level in the less stress-reactive sheep immediately after shearing and 3 h later was probably due to increased cleavage of CBG prompted by the increased neutrophil elastase and release of free cortisol which negatively feedback on the central activation of stress system. Also, Lin et al. (2009) hypothesized that human CBG exists in different conformational states with different steroid binding affinity. Therefore, we cannot exclude the possibility for existence of different conformational states of CBG in the more stress-reactive and less stress-reactive sheep. Also, CBG proteolysis was reported to coincide with an increase in pro- and anti-inflammatory plasma cytokine levels (Hill et al., 2016). Corticosteroid-binding globulin is a biomarker of inflammation onset and severity in female rats), and it is well documented that neutrophils mediate cytokine production and inflammatory response (Cerutti et al., 2013; Mócsai, 2013; Henry et al., 2016).

However, our interpretation concerning the association between cortisol level and N/L ratio in less stress-reactive and more stress-reactive sheep is not in agreement with the opposite association between these parameters in BSDP and Ile de France sheep. These seemingly conflicting results can be reconciled with the reported temperature sensitivity of CBG and increased release of free cortisol in response to increasing temperatures within the physiological range (Henley & Lightman, 2011). It is well known that milk breeds generate more metabolic heat due to high milk production (Polsky & von Keyserlingk, 2017). Besides, shearing was shown to increase body temperature (Sanger et al., 2011). Therefore, it is logical to expect higher physiological temperature ranges in BSDP sheep, especially following shearing, when sheep fleece is removed and the animals are exposed to direct solar radiation. This view is consistent with the sharp decline of cortisol level at 3h following shearing in BSDP sheep that could be due to temperature-induced increase of CBG proteolysis and negative feedback inhibition of the stress system by the expected increase of plasma free cortisol level.

## Conclusion

We did not find proportional increase of N/L ratio with increase of cortisol magnitude in sheep of Ile de France breed during exposure to shearing stress. The rate of neutrophil-to-lymphocyte ratio increase in the more stress-reactive sheep was less than that in the less stress-reactive sheep. There were clear cut interbreed differences in the association between N/L ratio and stress-reactivity following exposure to shearing stress.

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*Received:* June, 15, 2018; *Accepted:* July, 25, 2018; *Published:* June, 30, 2019