

## RELATIONSHIP BETWEEN CHANGES IN THE FASCICULAR ZONE OF THE ADRENAL CORTEX AND AGE IN FIGHTING BREED (*BOS TAURUS*) IN DIFFERENT STRESS SITUATIONS

Relación entre los cambios de la zona fascicular de la corteza adrenal y la edad en ganado de lidia en diferentes situaciones de estres

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### RESUMEN

Se han utilizado veinte bovinos de raza de lida que fueron divididos en cuatro grupos de acuerdo con la edad y el tipo de lidia, con el fin de estudiar el posible efecto de diferentes situaciones de estrés sobre la morfología y la función de la corteza suprarrenal. Los grupos establecidos fueron los siguientes; Grupo I (hembras lidiadas en una plaza de toros de tientas de 3 años), Grupo II (machos lidiados en una plaza de toros portátil de 3 años), Grupo III (machos lidiados en una plaza de toros de 4 años,) y Grupo IV (hembras lidiadas en una plaza de tientas de 8 años). Se obtuvieron muestras de sangre y la glándula suprarrenal de todos los animales. Los valores de cortisol se obtuvieron de muestras de sangre. Los estudios estructurales y ultraestructurales se realizaron de la glándula suprarrenal. En el análisis estructural de la glándula adrenal se estudió la proporción de las diferentes zonas de la corteza adrenal y las características de las células de la zona fascicular. Los resultados muestran una mayor liberación de cortisol detectado en el grupo de hembras de mayor edad (grupo IV). Una mayor proporción de zona fascicular se detectó también en este grupo y significativas diferencias ( $p < 0,05$ ) se encontraron con respecto a los otros grupos. Además, los espongocitos de la zona fascicular en las hembras de edad avanzada presentan un mayor número de liposomas, estadísticamente significativo ( $p < 0,05$ ), que los otros grupos. Esto podría significar que los animales más viejos pueden tener una mayor capacidad de

síntesis de cortisol. Es posible un aumento de la corteza adrenal como consecuencia de las situaciones de estrés recurrentes sufridas por estos animales y se podría considerar como un indicador morfológico de estrés crónico.

**Palabras clave:** Stress, Bienestar animal, Glándula adrenal, ganado de lidia, cortisol.

## ABSTRACT

In order to study the possible effect of different stressful situations on the morphology and function of the adrenal cortex, twenty fighting breed were divided into four groups according to age and fighting characteristics; group I (3 year old females fought in a farm bullring), group II (3 year old male fought in a portable bullring), group III (4 year old males fought in a public bullring) and group IV (8 years old females fought in a farm bullring). Samples of blood and adrenal gland were taken from all the animals. Cortisol values were obtained from blood samples. Structural and ultrastructural studies were realized from adrenal gland. In the structural analysis of the adrenal gland the proportion of the different zones of the adrenal cortex and the characteristics of the cells of the fascicular zone were studied. The results show a higher release of cortisol detected in the group of old females (group IV). A higher proportion of fascicular zone was also detected in this group and significantly differences ( $p < 0,05$ ) were found with regard to the other groups. In addition, spongiocytes of the fascicular zone in older females presented a higher number of liposomes statistically significant ( $p < 0,05$ ) than the other groups, which could mean that the older animals could have a higher capacity of cortisol synthesis. It is possible the enlargement of the adrenal as consequence of recurrent stress situations suffered by these animals and could be considered as a morphological indicator of chronic stress.

**Key words:** Stress, Animal Welfare, Adrenal gland, Cortisol, Cattle, Fighting breed.

## INTRODUCTION

Fighting breed (*Bos taurus*), is a autochthonous bovine breed selected over centuries for its bravery, fierceness and aggressiveness. Animals are brought up in large extensions open countryside with minimal humane contact, making them easily animals subject to stress (Zavy *et al.*, 1992; Grandin, 1997). A major neuroendocrine mechanism in a stress reaction is the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Genuth, 2001; Koko *et al.*, 2004), resulting in a rapid increase in circulating adrenocorticotrophin (ACTH) and subsequent rise in glucocorticoids and/or catecholamine, which are critical for successful adaptation (Dallman *et al.*, 1992). These increases are the front-line endocrine mechanisms to defend the organism against the stressful conditions. Stressful stimuli result in release of ACTH from the pituitary, which stimulates the cells of the fascicular zone of the adrenal cortex to increase synthesis and secretion of cortisol (Axelrod y Reisine, 1984).

Therefore, many authors relate the cortisol with the stress response in animals, and its levels will depend on manipulation, the length of the same and any previous experience of the stress factor (Möstl y Palme, 2002; Illera *et al.*, 2007).

In bovine, the basal value of cortisol in animals at rest varies between 0.5 and 9 ng/ml according to the race in question (Grandin, 1997). In fighting bulls, such studies in animals at rest are scarce because of the difficulty involved in handling them, although in animals which have not yet been experienced fighting basal vary values between 6.0 ng/ml (Sánchez *et al.*, 1996) and 13.34 ng/ml (Gil-Cabrera *et al.*, 2005). Many handling situations in animals, such as taking blood samples, vaccination, transport or shoeing, provoke marked signs of adrenal cortex activity. Such responses exist from birth and are maximal at times just before transportation (Illera, 2005). Cortisol levels increase in any situation in which animals are handled and vary as a function of the time and type of stressing situation

(Möstl y Palme, 2002; Sánchez *et al.*, 1996). In fighting bulls, these levels increase sharply when they enter the bullring, when an animal will show signs of aggressiveness or fight (Illera, 2005). The mean level of cortisol in bulls after bullfight is 26.98 ng/ml (Gil-Cabrera *et al.*, 2005) and in females of the same race these levels may reach 317.6 ng/ml (Sánchez *et al.*, 1996). In fighting cows sacrificed in abattoirs the values depending on the waiting time and values of up to 446 ng/ml have been recorded and a elevate individual variability has been found (Seva *et al.*, 2007).

The fascicular zone is a central part of the adrenal cortex formed by cords or fascicles of *spongicytes*. They have a polyhedral shape a centrally placed nucleus and a cytoplasm containing abundant lipid droplets. Ultrastructurally, the smooth endoplasmic reticulum is the most characteristic organelle, with a moderately developed Golgi complex and abundant mitochondria with tubular crest (Dellman, 1994; Fawcet, 1995; Bernabé *et al.*, 2004). The lipid droplets can be seen by electron microscope (Matsukuma, 1981), by means of which different degrees of electro-density depending on the maturation or secretion phases can be identified (Bernabé *et al.*, 2004). The proportion of adrenal cortex and medulla is similar for different species and small differences in the proportion of different zones of cortex have been described (Fawcet, 1995; Greco y Stabenfeldt, 2003). Variations in the structure of this fascicular zone affecting the size have been described in different species and in response to different stimuli both acute as chronic (Koko *et al.*, 2004; Milosevic *et al.*, 2005; Kmiec *et al.*, 2006). Persistent ACTH release, as occurs with chronic or repeated exposure to stressors, causes initial hypertrophy and then hyperplasia of the fascicular zone in adrenal gland (Dallman *et al.*, 1992). Therefore, enlargement of the adrenal cortex has been used as a morphological indicator of chronic stress (Kirilov *et al.*, 2003; Ulrich-Lai *et al.*, 2006)

The purpose of the present study was to determine the cortisol levels in blood and structural and ultrastructural modifications of the fascicular zone of the adrenal gland in fighting breed in different stressing situations. In this sent our intention is to help clarify the relationship possible between stress and physiology and morphology adrenal with the age and the sex in a breed that provides a good model of aggressiveness and could be extensive reared cattle.

## MATERIAL AND METHODS

*Animals:* Twenty fighting breed (*Bos taurus*) were divided into four groups of five animals. Group I (3 year old females fought in a farm bullring), group II (3 year old male fought in a portable bullring), Group III (4 year old males fought in a public bullring) and group IV (8 years old females fought in a farm bullring). At the end to fought all animals were sacrificed in bullring, type of sacrifice exclusively authorised in Spain and France by the European Community legislation (Lisbon Treaty, art.13: [...] *while respecting the legislative or administrative provisions and customs of Member States relating in particular to religious events, cultural traditions and regional heritage*).

*Blood samples:* blood samples were obtained from the cadavers in Aquisel® tubes without coagulant and kept at 4 °C for their transportation to the laboratory. The time between death and collection blood never exceed 5 min. Processing was carried out in the Veterinary Teaching Hospital of the University of Murcia. The samples were centrifuged in a Nahita® 2652 Model centrifuge (7 minutes at 2000 g) and the sera were extracted. Lastly, the level of cortisol was determined by commercial ELISA kit (EIA Radim®, Pomezia, Rome) and microtitulation plate reader, Powerwave XS® (Biotek Instruments GmbH, Germany).

**Adrenal samples:** Samples of the left adrenal gland were collected during necropsy. Fragments ( $0.5 \text{ cm}^3$ ) of the same were immersed in 10% buffered formol for subsequent processing and morphological study. In addition, fragments of approximately  $1 \text{ mm}^2$  were immersed in McDowell fixing liquid for ultrastructural study.

For adrenal morphological studies samples fixed in formol were included in paraffin using an automatic Histokinette 2000<sup>®</sup> tissue processor (Reichert Jung, Germany), fitted with a C6960 type external vacuum pump. From the blocks obtained  $5 \mu\text{m}$  thick sections were obtained with a microtome Biocut 2030<sup>®</sup> (Leica, Germany). After deparaffination and hydration, they were stained with haematoxylin-eosin and Masson trichromic. After final dehydration they were mounted with Eukitt<sup>®</sup> and observed under an Axioskop 40 Zeiss<sup>®</sup> optical microscope with a fitted camera and using the SPOT<sup>®</sup> software. Proportions of fascicular zone were calculated with respect to the total glandular area, taking 20 measurements of each gland expressed as a percentage. Every section was analysed under the final magnification of 50 X using the semiquantitative software Lucia 3.1.

The ultrastructural study was carried out with the samples fixed in McDowell liquid. These were rinsed in a cacodylate and saccharose solution before inclusion in Epon. From the samples semi thin ( $0.5 \mu\text{m}$ ) sections were obtained using a Reichert-Jung<sup>®</sup> microtome. These were then stained with toluidine blue to observe the lipid droplets, which were counted (in 100 cells per animal). Ultramicrotome sections of 15-150 nm were also made, using Osmium tetroxide as contrast to visualise the lipids. The sections were gathered on copper grids for observation by transmission electron microscope (Philips Tecnai 12<sup>®</sup>) equipped with a digital image capture system.

**Statistical analysis:** For the statistical analysis of the cortisol in the different groups an

ANOVA was used, the results of which were confirmed by a hypothesis contrast of the population means of the different groups. As regards the data related with the structure of the adrenal gland an ANOVA was made to ascertain whether the difference between the proportions of cortex and adrenal medulla were significant, as between the different zones of the adrenal cortex. Then a hypothesis contrast was made to determine whether significant differences existed between the proportions of the fascicular zone in the different groups. For this statistical analysis the Microsoft Excel<sup>®</sup> program was used with the theoretical data for formulae and tables (Nortes *et al.*, 1993)

## RESULTS

The cortisol values (ng/ml) obtained for the blood analyses of each of the animals show an elevated variability individually (Fig. 1). Although the mean is higher for the oldest animals (group IV) statistically no significant differences between the cortisol values of the different groups were observed.

In the fighting breed animals studied the adrenal glands were similar to those described

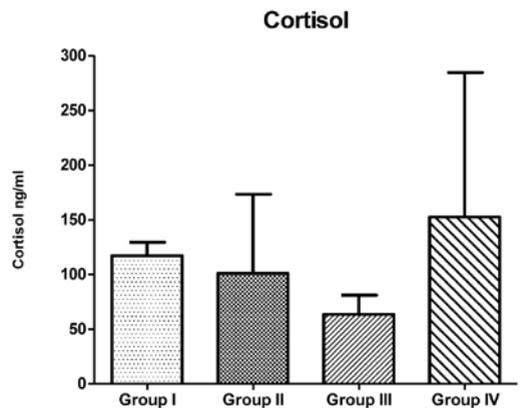
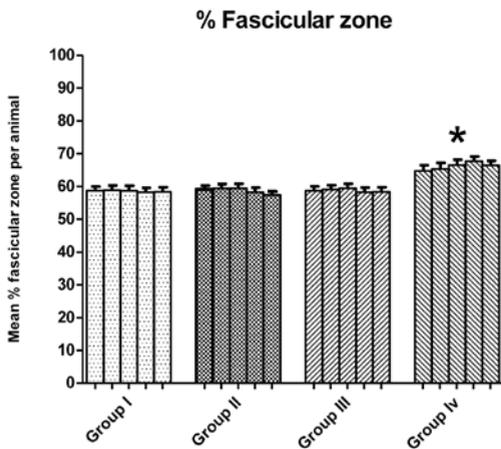


Figure 1. Values of Cortisol in the different groups.

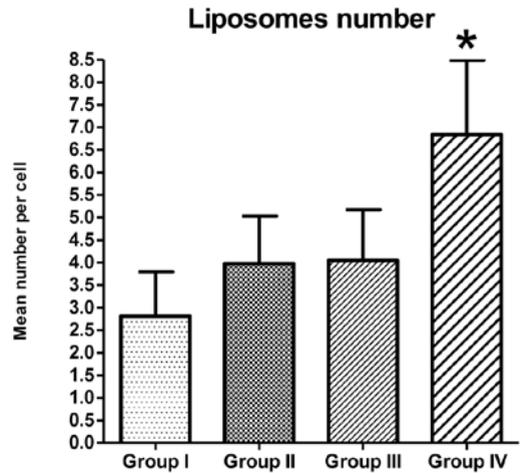
in the literature for others bovines. The normal colour of the gland was brown, although those of group IV were slightly more yellow. The intermediate zone of the adrenal cortex was the most studied since it is the site of cortisol secretion. The percentage represented by the fascicular zone was statistically significant ( $p < 0, 05$ ) greater in group IV than in the other groups (Fig. 2).



\* Significantly differences ( $p < 0, 05$ ).

Figure 2. Percentage of fascicular zone in the different groups.

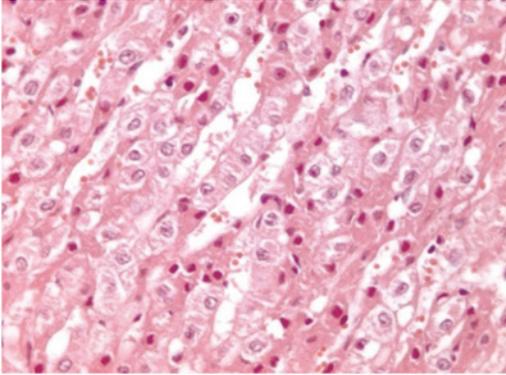
In the structural study, cells of the fascicular zone appeared forming cords with one side in contact with blood capillaries (Fig. 4). Hypertrophy and hyperplasia cellular were observed. The capillaries were pointing towards the interior of the gland, where the steroidal substances of lipid origin are released. These substances appear in form of round structures of varying size which stained most intensely with toluidine blue (Fig. 5). The number and size varied between groups, being statistically significant ( $p < 0, 05$ ) greater in group IV ( $6.75 \pm 1.04$ ) than in the other groups, as it can be seen in Fig. 3.



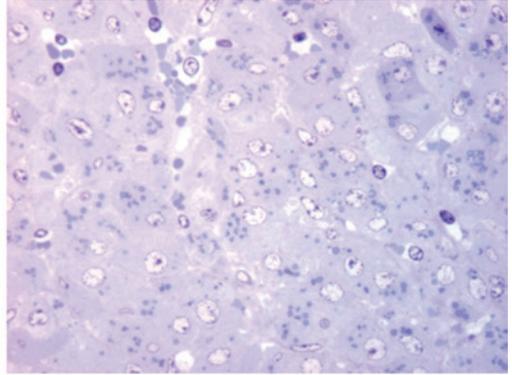
\* Significantly differences ( $p < 0, 05$ ).

Figure 3. Mean number of vacuoles in cells of fascicular zone in the different groups.

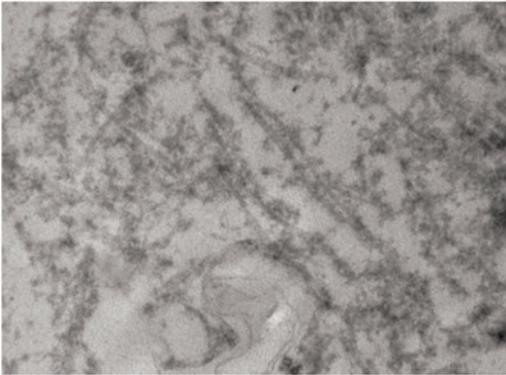
In the ultrastructurally study, spongiocytos showed a polyhedral shape, with a rounded nucleus centred in the cytoplasm, which was clearer in colour due to the presence of vacuoles. The most abundant cytoplasmic organelles as seen by electron microscopy was the smooth endoplasmic reticulum (Fig. 6), accompanied by a multitude of mitochondria with digital shape crests (Fig. 7). The cells of the fascicular zone in the animals of all groups generally contained an abundance of lipid vacuoles or liposomes in different stages of maturation and secretion, resulting in different degrees of electro-density. Some images corresponded to the formation process, whereby liposomes bind to the lipoproteins at the periphery of the lipid droplets (Fig. 8). The number and size of these liposomes (Fig. 9) varied with individual groups showing maximal expression in the oldest animals (group IV). The number of liposomes was statistically significant ( $p < 0, 05$ ) for group IV (Fig. 3).



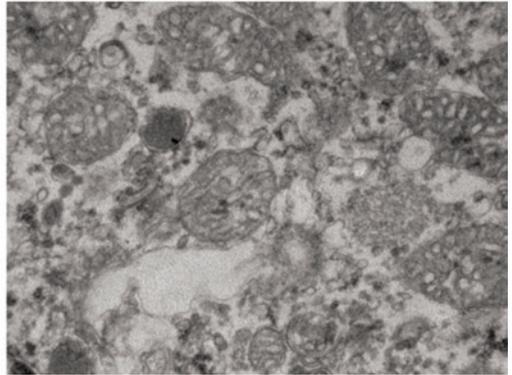
**Figure 4. Fascicular zone.  
Group IV. (H-E x 400A).**



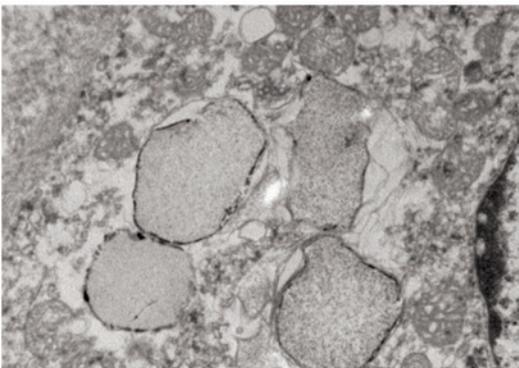
**Figure 5. Fascicular zone.  
Group IV. (A-T x 630A).**



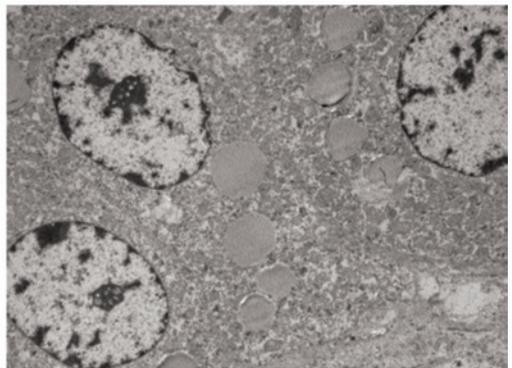
**Figure 6. Detail of smooth and rough  
endoplasmic reticulum (x 26,000).**



**Figure 7. Mitochondria with digital  
shape crests (x 26,000).**



**Figure 8. Formation of liposomes  
(x 15,000).**



**Figure 9. Liposomes in different  
stages of maturation (x 4,400).**

## DISCUSSION

In this study, the cortisol values in the blood of fighting breed in different situations of stress, age and sex were measured. Results show for this breed in stress situations higher values than animals without manipulation and other bovine races studied (Grandin, 1997; Agüera *et al.*, 2001; Gil-Cabrera *et al.*, 2005; Illera *et al.*, 2007). Thus, in this way cortisol levels increase in fighting breed exposed to manipulation or stressing situations and could be used as indicators of the level of stress acute (Agüera *et al.*, 2001; Sánchez *et al.*, 2003; Illera *et al.*, 2006). Plasma levels of glucocorticoids are a good indicator of stress response intensity, particularly in its acute phase (Pignatelli *et al.*, 1998), although the appearance of chronic stimuli of stress also could provoke the release of cortisol and androgens from the adrenal cortex (Terio *et al.*, 2004; Woods y Judd, 2007; Woods *et al.*, 2008) and it could be possible an interference in both responses in stressing situations as described this study. The response to stressing situations is high variable and transportation is probably the most stressing situation for fighting breed (Illera, 2005) although slaughter in the abattoir is mentioned by other authors (Seva *et al.*, 2007). In our study, only transported by lorry were animals of groups II and III and the former showed the highest mean cortisol levels although were younger (group II). This is due to legislation, the bulls of this group II must arrive at their destination 6 hours before being spectacle (Spanish Royal Decree 145/1996), while destined for major bull fights (group III) must be allowed to rest in pens for 24-48 hours. In this sense the animals of group II could have higher levels of cortisol by effect of transport since they came directly from the lorry to the bullring and both acute and chronic response could be working. On the other hand, cortisol levels obtained in this study were still lower than those described in fighting cows sacrificed in abattoirs (Seva *et al.*, 2007), which under-

lines the stress which animals in these places. In addition, they are higher levels even that experienced by fighting bulls in the bullring (Gil-Cabrera *et al.*, 2005). In this sense, for this bovine breed could be more stressful be enclosed in a block of a building than be in the outdoor arena during the fought.

Cortisol release is not a reflex mechanism each individual of the fighting breed may be capable to relate different stimuli with the different situations that have caused stress during their life for adapting their response to them (Illera *et al.*, 2007). The mean blood cortisol levels differ in each group, although not to a statistically significant degree. This could be due to the fact that there is a wide individual variability of cortisol release levels in the groups of animals studied (Greco y Stabenfeldt, 2003), providing very high typical deviations higher than other breed. Bearing in mind other studies (Sánchez *et al.*, 1996; Gil-Cabrera *et al.*, 2005) it would be expected that with a larger population (providing greater representative) the observed heterogeneity for the different groups would be attributable to different stressing factors studied. Therefore, a high individual variation in cortisol production was verified, this fact would be related by genetic selection process of fighting breed whole the behaviour is the fundamentally character required.

The proportion of adrenal cortex and medulla in fighting breed is similar to the value described for bovine species in general (Dellman, 1994; Greco y Stabenfeldt, 2003), there were no statistically significant differences between the different groups studied. However, the fascicular zone of group IV (older animals) appeared to be more developed than for the other groups (approximately 10%). In addition, these animals had highest blood levels of cortisol, so that they would have a greater power of synthesis and release of this hormone when confronted with stressful stimuli (Illera *et al.*, 2006). It has been described how the fascicular zone changes in the face of acute stressful situations (Gesi *et al.*,

2001), becoming smaller with acute stress stimuli (Koko *et al.*, 2001) and increasing in size as the stress becomes more chronic (Kirilov *et al.*, 2003; Milosevic *et al.*, 2005; Ulrich-Lai *et al.*, 2006). This fact was reflected in our study, where the 8 year old animals (group IV) would have been exposed to frequent stressing situations in their lives annual veterinary checks, synchronisation of heat, or vaccination programmes (Illera, 2005), some which events would have been faced once only in the lives of the other groups. Thus, for the fighting breed enlargement of the adrenal cortex could be used as a morphological indicator of chronic stress as have been described for other species (Kmiec *et al.*, 2006; Ulrich-Lai *et al.*, 2006).

Spongicytes of the fascicular zone of the adrenal cortex showed typical structures of steroid hormone-producing cells (Genuth, 2001; Greco y Stabenfeldt, 2003) and showed similar characteristics in all the groups studied. Particularly of note in these was the abundance of lipid-like structures in the cytoplasm, although the exact number and size varied between groups. They could be visualised by toluidine blue staining of varying intensity in the semi thin sections and not as optically vacant structures as might be expected, since these samples were fixed with osmium tetroxide and there was no loss of lipids during processing (Aguas y Nickerson, 1981; Wigglesworth, 1981). In other species, no differences in the degree of vacuolisation were observed in the spongicytes in the face of chronic stress situations (Terio *et al.*, 2004). Ultrastructurally, these corresponded to liposomes in different stages of maturation since they showed different degrees of electro-density, increasing towards the periphery (site of the liposomal matrix) and consequence of the binding of lipids and liposomes necessary for the final formation of cortisol (Matsukuma *et al.*, 1981; Tóth *et al.*, 1997). Although this lipid-liposome binding is rarely seen because of its short duration (Tóth *et al.*, 1997), it was frequent in the older animals of group IV (old-

er animals), perhaps reflecting the great cortisol formation activity of these animals, which showed the highest quantity of liposomes and cortisol levels, although slightly lower than in other females of fighting species (Sánchez *et al.*, 1996). These observations may be related since, due to the frequent handling these animals would have experienced, their cells would be constantly synthesising cortisol and releasing it to the blood (Tóth *et al.*, 1997), the images of lipid droplets in the vessels being more frequent in the fascicular zone of these animals than in other groups. There seems, then exist a capacity to relate the different stimuli to past situations that generated stress, permitting the animals to adapt their response (Illera *et al.*, 2007). Thus, faced with a stimulus, the response of these animals would be more immediate and would attain higher levels than in other animals. In contrast, the animals of the other group, bulls destined for bullfights, are subjected to minimal handling since they are free of blood test and not frequently enter in the installations of manipulations (Sánchez-Belda, 2002). Morphologically, the cells of the adrenal gland of these animals show lower quantities of liposomes and those which they possess are smaller in size, suggesting they are less prepared for producing cortisol and therefore for initiating an immediate response. In this sense another argument supporting this greater capacity of cells the animals of group IV to release cortisol is that they presented higher values than the animals of with group II, even though the conditions in which they fought were the same.

In conclusion, in this bovine breed which has been selected for its behaviour (bravery, fierceness or aggressiveness) and is breed in environments with little human contact, the older animals have been more constantly subjected to stress situations. We have observed in these older animals as the fascicular zone attains greater size and ultrastructurally spongicytes show a higher number of liposomes which could mean that the older animals could have a higher capacity of

cortisol synthesis as consequence of recurrent stress situations. Therefore, for this cattle could be considered the enlargement of the adrenal as a morphological indicator of chronic stress, although a greater number of studies are necessary in this sense to probe our findings.

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