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Adaptive Metabolic Responses in Females of the Fighting Breed Submitted to Different Sequences of Stress Stimuli

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SÁNCHEZ, J. M., M. J. CASTRO, M. E. ALONSO AND V. R. GAUDIOSO. *Adaptive metabolic responses in females of the fighting breed submitted to different sequences of stress stimuli.* PHYSIOL BEHAV **60**(4) 1047–1052, 1996.—The objectives of this study were to evaluate the stress reaction and the metabolic adaptive effort in females of the fighting breed when submitted to different manipulation sequences. Nine 4- to 8-year-old bovine fighting breed females were slaughtered to establish the basal levels of different blood parameters. A study was, then, conducted to examine the metabolic response in 30 2-year-old females, divided into 3 groups of 10 animals and submitted to different manipulations in each group: restraint-"open-field"-restraint, "open-field"-restraint, and transportation-restraint-"open-field"-restraint. The basal levels of the different blood parameters found were, in general, similar to the levels for cattle given in the literature. All the manipulations resulted in increases that were statistically different ($p < 0.001$) from basal levels, in terms of both cortisol plasma levels and the Specie Specific Experimental Response to Stress index (SSERTS). The stress of restraint (and the prior manipulations) seemed to mask the stress associated with the open-field and transport situations. In general, animals responded to 13 of the 15 parameters examined in the various experimental manipulations.

Animal welfare Cattle Cortisol Fighting breed SSERTS index Stress

THERE have been numerous approaches used to evaluate stress and adaptive metabolic effort in animals submitted to different stimuli, although all of them show extensive and well recognized limitations [2,3,6,8]. Moberg [23] suggests that the traditional methods for measuring individual discrete physiological responses, such as heart rate, corticosteroid concentrations, etc., should be replaced by examination of the global effects on animal welfare (reproductive, immune, and metabolic effects). The effects on reproduction and the immune system should be studied in animals subjected to middle- and long-term stress, and the metabolic effects should be studied to quantify the response to acute stress.

The Specie Specific Experimental Response to Stress index (SSERTS), described by Hattingh [14], is based on the percentage variation of some parameters altered during the stress response, where the greatest change observed for any one variable in the animal species studied is taken as 100%. Taking into account the entire range of variables, a global idea of the metabolic state of the animal is obtained, which, according to Moberg [23], is the best way of studying the well-being of the animal.

The response to acute stress is obtained by global measurement of several parameters that reflect the hormonal and metabolic state of an individual at a given time. The result obtained, from zero to 100, shows mathematically and gradually the effort that each animal had to exert to overcome a stressor. In short, the SSERTS index measures the internal stress effects [14] that can be observed in:

- Changes in the fluid balance and/or in the proportion of electrolytes;
- changes in the general use of substrates;
- changes in the concentration of primary stress hormones; and
- changes in the concentration of other blood parameters.

On the other hand, a succession of stressful stimuli can produce an increase in adrenal sensitivity similar to that described by Lilly and Gann [21] and Lilly et al. [22] in dogs and by Kenny and Tarrant [18] in cattle, who found progressive increases in the plasma levels of cortisol and glucose when young bulls were submitted to a sequence of stimuli (restraint, truck confinement, transport, etc.).

The objectives of this study were to determine the basal values for some of the most used plasma parameters and to evaluate the stress reaction and the adaptive metabolic effort in females of the fighting breed when submitted to different, particular, and controlled manipulation sequences. This breed was chosen because it provides a good model of aggressive and extensively reared cattle.

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MATERIALS AND METHODS

Control Animals

To establish the basal levels of the blood parameters considered, a control group of nine adult females (4 to 8 years old) that belonged to three different farms of the "Unión de Criadores de Toros de Lidia (UCTL)" were killed with a rifle shot to the brain, fired from a car the animals were accustomed to seeing in their pasture. The samples were collected immediately after the animal died (approximately 30 s from shooting to sampling). Consequently, we can assume that the samples represent basal values. The animals were all in distinct and separate locations, and were killed between 11.00 and 13.00 h on 3 consecutive days.

Experimental Animals and Manipulations

Thirty 2-year-old nonpregnant fighting breed cows, belonging to the same farm and of a similar body weight (approximately 150 kg), were randomly divided into 3 groups of 10 animals each. The animals were subjected to the following manipulations:

- Group 1: Restraint in a cattle crush with a blood sample taken from the jugular vein. Subsequently, a 5-min open-field test was carried out followed by a second restraint in a cattle crush to take a further blood sample.
- Group 2: A 5-min open-field test followed by restraint in a cattle crush with a blood sample taken from the jugular vein.
- Group 3: The 10 animals from this group were randomly divided into 2 groups of 5 animals each, and were transported by truck for half an hour, followed by restraint in a cattle crush with a blood sample taken from the jugular vein. Following this, animals were exposed to a 5-min open-field test and another restraint in a cattle crush for a blood sample to be taken. Each subgroup of 5 animals was transported on 2 consecutive days.

These manipulations were chosen because restraint and transport are two of the most frequent handling procedures in this breed, and open-field testing is commonly used in behavioral research.

All of the experimental manipulations were carried out on 4 consecutive days from 11:00 to 13:00 h, under similar weather conditions, beginning with Group 1. The animals of each group were brought from pasture fields to small pens at 10:00 h and, after 11:00 h, isolated from companions just prior to each manipulation.

The animals were only restrained during blood sampling (approximately 1 min) and the manipulations prior to placing and restraining them in the cattle crush lasted approximately 10 min when restraint was the first manipulation (because it was necessary to isolate each animal from companions), and less than 5 min when restraint followed other manipulations. All the open-field testing was carried out in one circular pen (16.9-meter diameter), new to the animals, with the floor base divided into 9 equal parts (i.e., using the method described by Kilgour [19]).

Using this protocol, blood samples were taken at the times of 5 different situations:

- Sample R: Restraint (first time in crush for females of Group 1).
- Sample OF-R: Open-field test and restraint (only time in crush for females of Group 2).
- Sample R-OF-R: Restraint, open-field, and restraint (second time in crush for females in Group 1).
- Sample T-R: Transportation and restraint (first time in crush for females in Group 3).
- Sample T-R-OF-R: Transportation, restraint, open-field test, and restraint (second time in crush for females in Group 3).

Analytic Methodology

For each sample, a minimum of 10 cc of blood was taken into heparinized tubes and immediately afterwards centrifuged at 1250 *g* for 10 min. Three 1-cc plasma aliquots were placed in Eppendorf tubes, frozen, and stored at −20°C. One of the frozen aliquots was used to determine cortisol levels. A second was used to determine the other plasma components and the third was kept in reserve in case it was necessary to repeat any of the analyses.

The plasma parameters studied were: cortisol, glucose, uric acid, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), triglycerides, total proteins, calcium, phosphorus, sodium, potassium, and chloride.

A cortisol kit (No. 1114 by Immunotech® International S.A., Luminy, Case 915, 13288 Marseille Cedex 9, France) was used to determine plasma cortisol levels. It is a radioimmunological competition assay. The intraassay ($n = 10$) and the interassay ($n = 15$) coefficients of variation were 6% and 7.2%, respectively.

A Hitachi-737® was used to determine the remaining parameters using Boehringer-Mannheim GmbH Diagnostica® (Sandhofer Strasse 116 D-68298, Manhein, Germany) reagents.

All of the samples were analyzed in duplicate and the mean was used.

SSERTS Index Calculation

The methodology described by Hattingh [14] was used to calculate the SSERTS, applying the following formula for each one of the 15 blood parameters studied:

$$\frac{Ev - Ac}{Mv - Ac} \times 100$$

where Ev = experimentally found value, Ac = parameter average in the control group, and Mv = maximum value found for this parameter in the 30 animals studied.

The SSERTS index for an animal is obtained by calculating the average of the values obtained using the above-mentioned formula for each blood parameter in this animal. The SSERTS index for a group of samples is the average of the SSERTS index values obtained for each animal within the group of samples.

Statistical Analyses

There were 6 treatment groups, samples taken from the controls and from the 5 manipulative situations.

One-way analyses of variance were made of the SSERTS index and of the plasma levels of cortisol among the control group and the 5 experimental situations. The differences between the means were tested using the F-Fisher-PLSD test (Planned Low Significant Difference).

In all the plasma parameters considered, the differences between the means in each experimental situation and the control group were tested using the F-Fisher-PLSD test.

All this was carried out using the Macintosh statistical program Statview 512+®.

Furthermore, a stepwise discriminant analysis [7] was made between the control group and the 5 experimental situations using all the plasma parameters considered. For this purpose, the software package, Biomedical Computer Programs (BMDP) of the University of California was used. The discriminant analysis is useful when data are grouped (as in our case, where we have 6 groups) because it provides classification functions (linear com-

TABLE 1

MEAN ± STANDARD DEVIATION OF PLASMA PARAMETERS IN THE CONTROL AND THE DIFFERENT EXPERIMENTAL MANIPULATION SITUATIONS

Parameters	Control	R	OF-R	R-OF-R	T-R	T-R-OF-R
Cortisol (nmol/l)	6.0 ± 6.81	243.4 ± 36.45‡	203.9 ± 41.71‡	293.1 ± 34.69‡	283.9 ± 58.25‡	317.6 ± 57.27‡
Glucose (mg/dl)	63.1 ± 7.52	121.6 ± 24.58*	150.7 ± 31.27†	184.0 ± 33.48‡	164.8 ± 54.34‡	236.4 ± 59.73‡
Uric acid (mg/dl)	0.62 ± 0.137	0.97 ± 0.149	1.01 ± 0.166	1.58 ± 0.614*	1.01 ± 0.373	1.97 ± 0.968†
Urea (mg/dl)	35.8 ± 14.21	27.0 ± 6.37	27.8 ± 5.99	21.4 ± 6.07	32.7 ± 8.94	34.1 ± 9.57
Triglycerides (mg/dl)	26.0 ± 5.33	18.6 ± 3.86*	17.3 ± 2.16*	19.0 ± 3.65*	19.0 ± 8.86*	21.1 ± 6.79
Proteins (g/dl)	7.4 ± 0.68	8.4 ± 0.45‡	8.0 ± 0.20	8.1 ± 0.43	7.6 ± 0.66	7.3 ± 0.53
Creatinine (mg/dl)	1.38 ± 0.192	2.00 ± 0.353†	1.88 ± 0.204†	2.22 ± 0.418†	2.00 ± 0.115†	2.02 ± 0.139†
AST (U/l)	86.0 ± 15.98	98.2 ± 30.53	101.5 ± 18.59	116.0 ± 35.61*	112.6 ± 18.03*	143.0 ± 26.96‡
ALT (U/l)	26.2 ± 3.56	37.8 ± 8.38*	39.9 ± 7.55*	38.1 ± 8.20*	37.7 ± 4.94*	39.9 ± 4.58*
CK (U/l)	532.9 ± 387.49	404.1 ± 186.35	721.1 ± 371.90	1648.5 ± 1183.03*	1500.2 ± 915.27*	3871.4 ± 1898.30‡
Calcium (mg/dl)	9.8 ± 0.61	10.1 ± 0.31	10.3 ± 0.44*	10.1 ± 0.18	10.4 ± 0.73*	10.2 ± 0.62
Phosphorus (mg/dl)	6.6 ± 1.01	6.5 ± 1.04	6.4 ± 1.15	5.4 ± 1.29	4.3 ± 1.17†	3.4 ± 0.97‡
Sodium (mmol/l)	150.0 ± 4.66	149.5 ± 3.37	152.8 ± 2.74	152.3 ± 3.06	152.8 ± 2.94	149.3 ± 3.01
Potassium (mmol/l)	5.3 ± 0.51	5.3 ± 0.44	5.6 ± 0.67	5.8 ± 0.57	6.0 ± 1.12*	5.8 ± 1.10
Chloride (mmol/l)	107.2 ± 5.26	94.3 ± 2.26‡	93.6 ± 3.03‡	91.9 ± 3.35‡	94.2 ± 1.69‡	92.9 ± 2.75‡

* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$, vs. controls.

bination of those plasma parameters that add the most to the separation of the groups, variables with discriminant power) and, at the same time, gives canonical variables (also a linear combination of those variables with discriminant power) that allow us to graphically represent the differences between groups. This way, the groups are graphically more or less close if their animals have or do not have similar levels in the biochemical parameters with discriminant power.

RESULTS

Tables 1 and 2 (for cortisol) show the means and standard deviations of the levels of different plasma parameters studied in the control group and in each of the experimental manipulation situations, and the comparisons between the control means and each of the experimental means.

There were increases ($p < 0.001$) in the plasma cortisol levels in each of the 5 experimental situations compared with the control basal levels, and there is a decrease ($p < 0.00$) in chloride concentration in each of them. Furthermore, increases ($p < 0.05$) in the plasma levels of glucose, ALT, and creatinine were found in each of the 5 experimental situations. Generally, although not always significantly so, there were increases in levels of uric acid, AST, CK, and calcium, and decreases in triglycerides and phosphorus. However, no significant variations in the plasma levels of either urea or sodium were found.

The levels of cortisol, in addition to being found to be greater ($F(5, 53) = 71.643$, $p < 0.001$) than the basal levels in all the experimental situations, present statistically higher ($p < 0.05$) values (Table 2) in the blood samples taken after the most prolonged manipulations (e.g., T-R-OF-R, T-R, and R-OF-R).

Table 2 shows the SSERTS index values of the control group and the 5 experimental situations ($F(5, 53) = 6.481$, $p < 0.001$); the controls were always lower ($p < 0.05$). However, there are no differences between the experimental situations.

Tables 3 and 4, and Fig. 1 show the results of the stepwise discriminant analysis carried out among the 5 experimental situations and the control group using all the plasma parameters considered. Only 4 blood parameters (cortisol, urea, CK, and chloride) have discriminant power among the 6 situations. On the other hand, the classification functions place 70.4% of the cases correctly. All 9 females of the control group also proved to be correctly placed, although there are some exchanges of elements among the 5 experimental situations. In any case, the discriminant analysis detects differences ($F(20, 150) = 15.399$, $p < 0.001$) among the different situations, except between samples R and OF-R (Table 3).

TABLE 2

COMPARISON OF MEANS ± STANDARD DEVIATION OF THE PLASMA LEVELS OF CORTISOL AND THE SSERTS INDEX AMONGST THE 5 EXPERIMENTAL SITUATIONS AND THE CONTROL GROUP

	Cortisol	SSERTS
Control	6.0 ± 6.81[a]	9.8 ± 10.39[a]
R	243.4 ± 36.45[b]	28.9 ± 9.46[b]
OF-R	203.9 ± 41.73[c]	29.2 ± 8.63[b]
R-OF-R	293.1 ± 34.69[d]	31.3 ± 11.21[b]
T-R	283.9 ± 58.25[d]	32.8 ± 13.55[b]
T-R-OF-R	317.6 ± 57.27[d]	37.8 ± 13.85[b]

Different superscripts in the same column indicate differences ($p < 0.05$).

TABLE 3

$F(4, 45)$ PARTICULAR MATRIX BETWEEN GROUPS RESULTING FROM THE STEPWISE DISCRIMINANT ANALYSIS CARRIED OUT ON THE BLOOD SAMPLES FROM THE 6 SITUATIONS (5 EXPERIMENTAL AND 1 CONTROL)

Trials	Control	R	OF-R	R-OF-R	T-R
R	77.45‡				
OF-R	67.40‡	1.49			
R-OF-R	116.15‡	4.89†	8.29‡		
T-R	83.92‡	2.66*	4.00†	3.69*	
T-R-OF-R	94.74‡	13.91‡	14.55‡	8.01‡	5.24‡

* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$.

TABLE 4

COEFFICIENTS, CONSTANT, EIGENVALUES, AND CUMULATIVE VARIANCE OF THE CANONICAL VARIABLES 1 AND 2 RESULTING FROM THE DISCRIMINANT ANALYSIS CARRIED OUT ON THE BLOOD SAMPLES FROM THE 6 SITUATIONS (5 EXPERIMENTAL AND 1 CONTROL)

	Canonical Variable	
Variable	1	2
Cortisol	−0.91627	0.06248
Urea	2.07944	−2.41186
CK	0.00370	−0.03908
Chloride	9.97034	−1.23997
Constant	−18.79615	6.07448
Eigenvalues	13.44400	1.28750
Cumulative variance	0.89961	0.98576

Table 4 illustrates the first two canonical variables obtained in the discriminant analysis. It includes their eigenvalues and the accumulated explained variance. The first variable explains 89.96% of the study's global variance and is defined, mainly, by the chloride and urea parameters with a positive sign and cortisol with a negative sign. This means that the co-ordinates that the different groups have in this variable will depend on the concentrations of those parameters in each one of the samples, and clearly separates the control group from the other 5 experimental situations, as can be seen in the bidimensional representation of the centroids (or mean values) of the different manipulations (Fig. 1). The second variable explains 8.62% of the analysis variance (Table 4) and is defined mainly by the urea and chloride parameters with a negative sign, and separates the sample group T-R-OF-R from the other groups (Fig. 1).

DISCUSSION

First we admit the problems of use as control group animals of different ages and farms from the experimental animals but, taking into account the large magnitude of the differences between the control and the experimental values, the problems associated with research into this breed, and the fact that the blood parameters we found were, in general, similar to the values in the literature for other breeds of cattle [10,13,16,26,27], we believe that the controls were adequate and the best available. In particular, the basal plasma cortisol level we found is similar to the basal values considered by Kaneko [16] and Grandin [12] for cattle.

However, the CK values referred to in the literature [26] are very different from the values we found in the fighting breed. This might be due to differences in the analytical techniques used. In our study, the CK activity was measured at 37°C, whereas Tarrant and McVeigh [26] did it at 25°C. Nevertheless, even taking into account the appropriate conversions, the values found in the fighting breed are still much greater. On the other hand, it should be kept in mind that blood samples were taken after killing the animals by rifle shot to the brain, and this could increase the CK values due to cranial shock. The shot destroys brain tissue and, as Kaplan and Pesce [17] point out, the brain has a CK concentration of 160 U/gr. However, it does not seem very probable that this could be reflected in the peripheral blood level, given that there is only a 30-s time lapse between the shot and the sampling. Another possible explanation

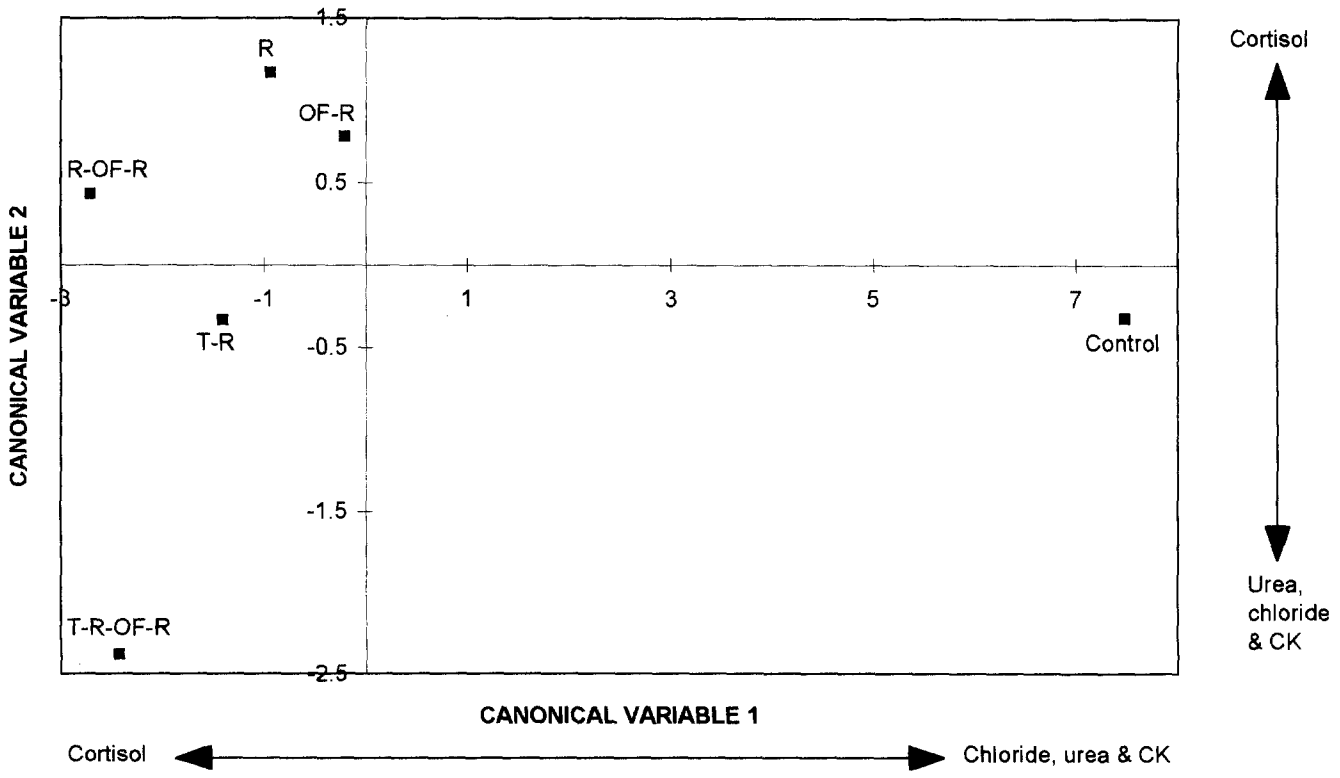

FIG. 1. Graphic representation of the mean of each treatment with respect to the canonical variables obtained by the discriminant analysis carried out between the control group and the 5 experimental situations.

for the high basal CK values we noted would be if the animals had a selenium and vitamin E deficiency; Allen et al. [1], Kramer [20] and García-Belenguer [11] point out that this can result in a rise in CK concentrations.

Significant increases in the plasma levels of cortisol were observed in each experimental situation compared with the basal control levels, implying that all the manipulations produced a stress reaction. Kenny and Tarrant [18] stated that cortisol perfectly reflects the stress response. Other authors [9,15,24] also find a direct relationship between the levels of 11-hydroxycorticosteroids and the intensity and/or duration of the stressful stimulus. In our study, we found a higher increase (even the differences among them were not significant) in the plasma level of cortisol after the more prolonged and complex manipulations (e.g., T-R, R-OF-R, and T-R-OF-R) (Table 2).

The presumably additive effect that the complexity of the manipulations seems to have on the degree of stress manifested by the animals could be used to identify the amount of causal responsibility in the stress reaction of each single manipulation. For example, if we take into account that in the case of transport it is necessary to restrain the animals to take the blood sample, it seems reasonable to conclude that the difference between the cortisol levels obtained after the transport-restraint (T-R) and those levels found after the restraint (R) alone is due to the actual transport. However, it is possible, and even likely, that the cortisol levels found after restraint do not reflect the stress produced by the restraint per se but are, rather, a result of the manipulations prior to the restraint, because the time between the moment in which the animal is restrained and the blood sample taken is not long enough to raise cortisol to high levels in the peripheral blood [24,25]. The same theory could be put forth in the case of transport, because the time lapse between the moment the animal leaves the truck and when the blood sample is taken is less than 5 min. Because there are differences between R and T-R, we can conclude that the stress produced in the fighting breed by half an hour of transport is higher than that produced by the manipulations prior to restraint alone.

Similarly, the females submitted to an OF test were also restrained for a blood sample to be taken but, again, the time lapse between the end of the test and the sampling was less than 5 min. In this case, the manipulations immediately prior to sampling the OF-R animals were the same as those endured by those animals only submitted to R. Conner et al. [4] and Dantzer and Mormede [5] point out that the display of behavior that is designed to control a new situation or that is used as an "escape reaction" decreases hormonal secretion. Therefore, this could explain why cortisol levels were less after OF-R than after R alone.

The cortisol levels of the R-OF-R animals were higher than those of the R and OF-R animals. It has to be noted that the time lapse between the first and the second restraint was only about 10 min. Thus, these animals, in addition to enduring the manipulations the animals in the other two groups endured, could have enough time for the stress produced by the first restraint to be reflected in peripheral blood cortisol levels. The stress produced by the R and the associated prior manipulations seems to be so strong that it masks any potential effects due to OF.

The most complex manipulation, T-R-OF-R, is the one that produces the highest levels of cortisol in the blood, even if there were no significant differences with the cortisol levels after R-OF-R and T-R, and this is very probably due to the fact that the stress is produced by 4 manipulations and that the entire duration of manipulation is longer than any of the other manipulation sequences.

The SSERTS indices show that there are clear differences between the control group and the other experimental situations, and that the adaptive metabolic effort among the 5 experimental situations is very similar. On the other hand, the discriminant analysis clearly shows significant differences between each one of the experimental situations and the control group, as can be seen first in the F matrix and, second, in the high percentage of correct classifications (100%) of the control group. However, the differences are not so clear between the different experimental situations because, although there are significant differences among them, in no one case does the percentage of correct classification reach 100%. This means that the animals' metabolic responses in the different situations can overlap. Thus, the most heterogeneous metabolic response is found in the T-R situation, which has only a 50% correct classification.

As can be seen in Fig. 1, the control group has a positive and high co-ordinate because its urea and chloride levels are high and its cortisol levels are low compared with the other experimental situations. On the contrary, the 5 experimental situations all have a negative coordinate and are quite close together because they have similar levels of biochemical parameters. The second canonical variable perfectly separates the T-R-OF-R manipulation because the urea and CK levels tend to be higher for this than for the other samples. The graph also illustrates that, as the manipulations become more prolonged and complex, the values of both coordinates are more negative.

As already mentioned, there was an obvious stress reaction in all the experimental situations. This fact, together with the discriminant analysis results and the increase in the SSERTS index for all the experimental situations, lead us to the conclusion that animals of the fighting breed require great internal adjustments when confronted with any manipulation, however simple. This would explain the great intrinsic reactivity of the breed, which is a result of centuries of breeding selection in search of a lower aggressive threshold.

Furthermore, the stress produced in the fighting breed by the R and the associated prior manipulations seems to be so strong that it masks any potential effects due to other manipulations, which must be taken into account in the design of further studies.

Finally, our results suggest that, to improve the welfare of the fighting breed, the usual handling manipulations should be minimized.

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