INTRODUCTION

The South African National Defence Force (SANDF), as any other defence force in the world, must train its forces continuously in order to be combat ready. Practising the use of weapons and the testing of new weaponry make up an integral part of this force preparation. Vast areas of land have been assigned for this purpose and the SANDF has a moral obligation to manage these areas optimally in order that once it is no longer needed it can be returned to the state for other usages.

Little factual information exists as to the extent and nature of the impact of military activities on the environment. It is often easy to see damage to the soil and plant component, but the long term effects are unpredictable. Furthermore, it is almost impossible to see the long term effect of military activities on the animals or the abiotic component of the environment.

The SANDF has thus embarked on a program where it supports research undertaken to estimate the influences of continual military activities on the environment in order to manage negative environmental impacts optimally. In order to facilitate this research the broad areas to be investigated were broken down in its various biotic and abiotic components. Examples of these are the effects of military activities on grass species, on trees, on wildlife and abiotic components. Information obtained from these projects is integrated into the management of every terrain in order to minimise the environmental impact of military exercises.

RESEARCH PROBLEM

Large numbers of wild animals roam the land used by the SANDF to train its forces. Although no changes in the natality, longevity, morbidity or mortality of these populations have been observed, a measurable parameter was sought to evaluate various military activity protocols in order to determine the best practise to be implemented to support the Military Integrated Environmental Management (MIEM) plan.

Where a pattern of disturbance of animal populations and resultant stressors have been prolonged over months and years, chronic physiological changes may take place [7, 6, 26, 41]. It has been postulated that these changes may lead to a disharmony within the hypothalamic-pituitary-axis, characterised by some as a discorrelation in endocrine control [44]. It is suggested that the disturbance of this central control of hormone secretion might affect the structure, storage function and activity of tropin hormone secreting cells in the hypophysis [13].

Radioimmunoassay is generally employed to study the bioactive peptides of the hypophysis in plasma [2, 44, 46, 61, 10, 48, 4, 58, 26, 41, 9]. Radioimmunoassays are cumbersome as a specific controlled procedure for the sampling of plasma is needed together with a wide variety of equipment to enable precise cooling and storage, all of which are not easily available under veld conditions [18, 12, 56, 3].

The precise estimation of blood hormone levels in wild animals is further confounded by the unavoidable need to restrain the subject in some way to enable the collection of samples, resulting in an alarm reaction accompanied by physiological responses indicative of acute stress [15, 30].

Plasma hormone concentrations, for example cortisol, change rapidly as part of this physiological response, making the measurement of these concentrations difficult to interpret [15].

Immunocytochemistry has been successfully used in visualising hormone secreting cells, including prolactin, somatotropin and ACTH, in the hypophysis of man [53], rats [58], primates [5], chickens [33], bats [1], mink [59] and sheep[42]. This technique has an advantage as samples do not have to be collected under specific controlled conditions, and tissues collected can be immediately fixed in a preservative [4]. It was thus decided to evaluate this technique and its potential to provide information on the effects of disturbance on wild animal populations. It was apparent to begin with that the quantification of changes seen would be difficult.

Computer assisted image analysis has been successfully employed to obtain prolactin and somatotropin immunoreactive sagittal area measurements in hypophyseal sections of turkeys. These measurements were expressed as a percentage to the total pituitary area [45]. If this technique could be successfully employed in animals to count the immunostained images, to measure the surface area of each image as well as to determine the proportion of total area stained, it would enable the quantification of changes in hormone secreting cells as a response to environmental factors, such as stress.

It was thus decided to evaluate the use of immunocytochemistry, combined with computer assisted image analysis, not only to visualise hypophyseal cells active for hormones, but also to attempt to determine the magnitude of the structural changes of the various hormone-secreting cells in the hypophysis of impala. In so doing a measurable parameter for determining the effects of military practises could be developed.

As a first step to test the combination of these two techniques, it was decided to compare the relative activity of prolactin secreting cells of the hypophyses of lactating and non-lactating wild animal populations by, firstly, statistically analysing the difference in the total number of all the images immunocytochemically stained for a specific hormone counted per animal. Secondly, to quantify the statistical mean of the surface area of all immunocytochemically stained images for a specific hormone. Thirdly to calculate the proportion of the total area stained. Then to compare the relative activity of somatotropin secreting cells of the hypophyses of male juvenile and adult impalas. Thereafter, if successful, the technique would be used to compare the relative activity of ACTH secreting cells of the hypophyses of free ranging impala with those of animals kept in an enclosure for various periods of time in order to establish if any significant differences from the mean ACTH immunoreactive image area, total images counted and proportion of the total area stained could be demonstrated.

Impalas (*Aepyceros melampus*) are abundant in many natural areas in South Africa as well as various SANDF controlled terrains, and often population control measures are necessary to prevent overgrazing [51]. This creates an opportunity to study the physiology of this species, including the effect of prolonged stressors, to obtain data which could be applied to the management of wildlife populations in general. Animals used in this investigation were culled as part of other projects undertaken by the National Parks Board in the southern areas of the Kruger National Park.

The objective of this study was thus to evaluate the use of immunocytochemical staining combined with computer assisted image analysis, as a measure of chronic stress levels in impala.

MATERIALS AND METHODS

Three trophic hormones were studied in impala of different age groups; free-ranging animals; and in newly captured impalas that were kept in confinement for up to 21 days.

Animals

Prolactin study

Twenty free ranging adult impala ewes were culled at the end of the breeding season, when lambs appeared to be almost weaned. The ewes were divided into two groups, lactating and non-lactating, by examining the udder for the presence of milk.

Somatotropin study

Nine, one year old impala rams, and seven, adult impala rams were culled, all of which were free-ranging.

ACTH study

Twenty three one year old impala rams were captured using a mixture of 2 mg etorphine hydrochloride[a] and 20 mg azaperone[b] using a Telinject GUT 50 remote darting system[c], a 2,5 ml syringe and a 30 mm collared needle. These animals were transported in an immobilised state

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[a] M99 - Logos Agvet, Private Bag X115, Halfway House, South Africa, 1685

[b] Azaperone - Kyron Laboratories (Pty) Ltd, PO Box 27329, Benrose, South Africa, 2011

[c] Telinject SA, PO Box 596, Jukskei Park, 2153

to an enclosure within 30 minutes after capture. Before release the effects of the etorphine hydrochloride were reversed using 6 mg of diprenorphine hydrochloride[d] given intravenously. These animals(n=23) were culled in the enclosure at different times after capture:

- On day 1 (n=7)
- On day 7 (n=8)
- On day 21 (n=8)

This was done in an attempt to establish any significant changes in terms of the mean ACTH immunoreactive image surface area, total immunoreactive images counted and proportion of the total area stained. All the samples collected from the other animal groups, non-lactating, lactating, adult and juvenile, were also included in this study as it was hypothesised that all these groups would be in various states of chronic stress.

In order to eliminate physiological responses due to acute stress, all animals were culled by a neck shot which severed the spinal cord [58].

Collection of hypophyses

Access to the brain was obtained by clamping the head in a vice and sawing a vertical cross section immediately caudal to the eyes downwards halfway into the skull with a post mortem saw (Figure 1). Thereafter, the top of the skull and the bulk of the brain were removed by a second, horizontal section, starting from the foramen magnum forward to join the first vertical cut (Figures 2 & 3). This exposed the floor of the cranial fossa (Figure 4), and the hypophysis could be dissected out using a scalpel and rat-tooth forceps.

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[d] M5050 - Logos Agvet, Private Bag X115, Halfway House, South Africa, 1685

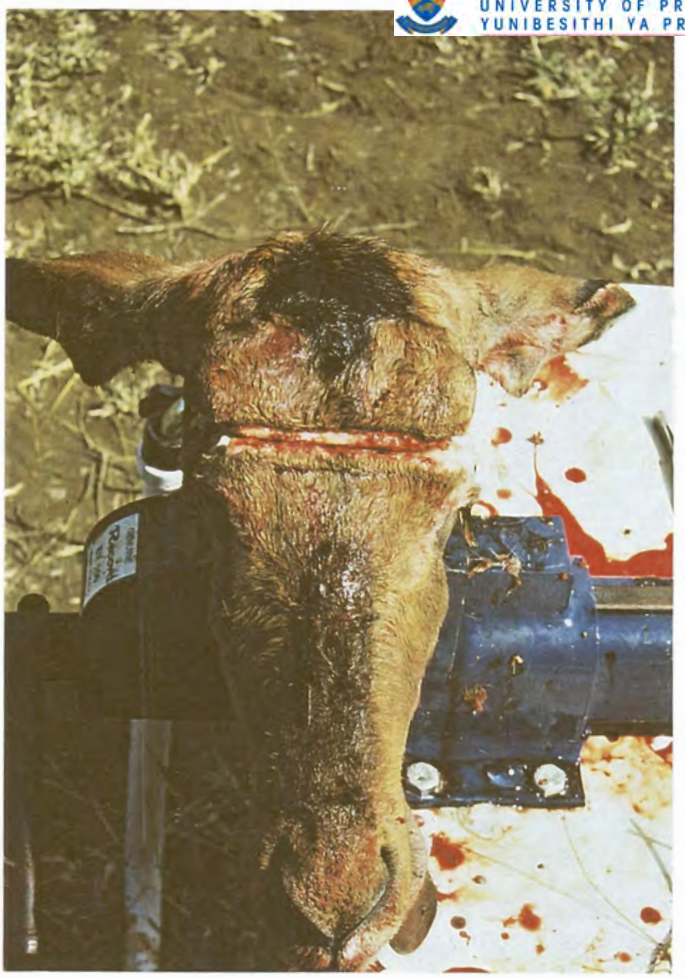

Figure 1. Vertical cross section immediately caudal to the eyes

Figure 2. Second horizontal section, starting from the foramen magnum forward to join the vertical cut

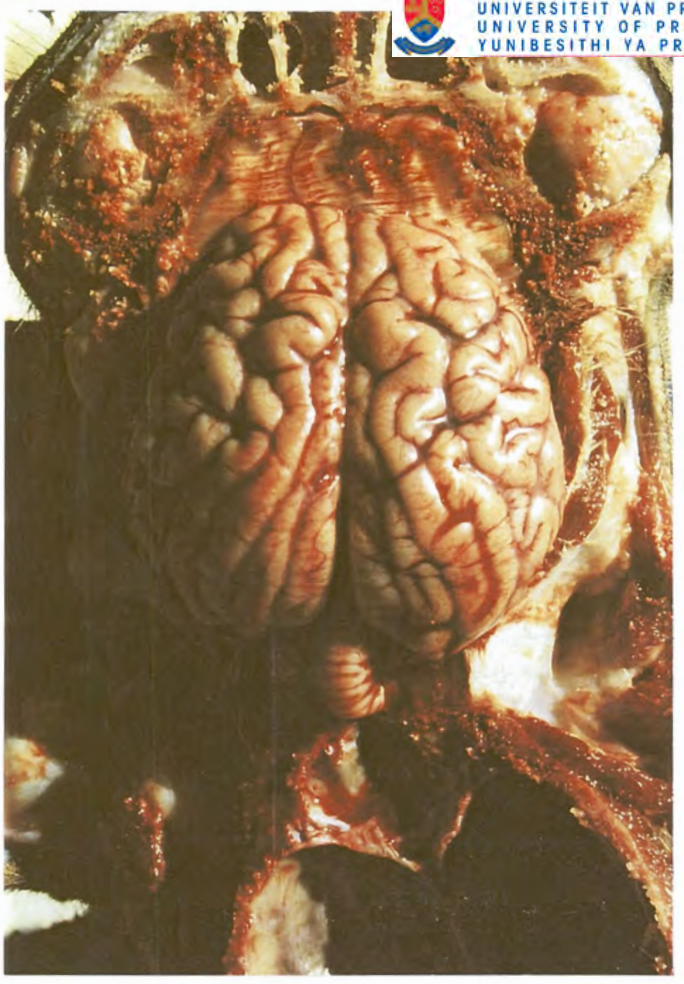

Figure 3. Bulk of the brain ready to be removed

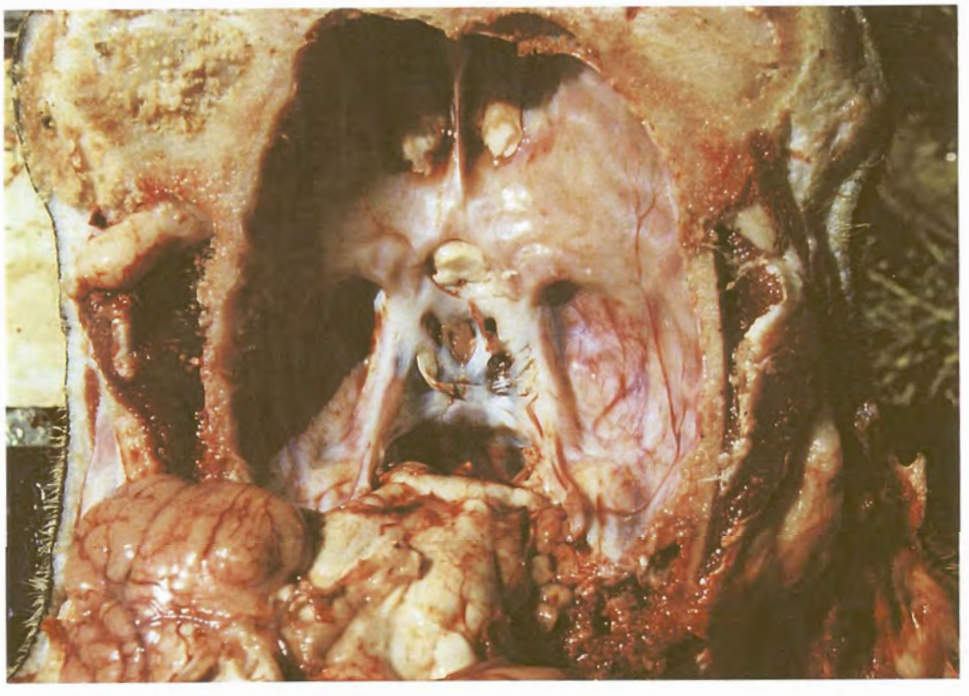

Figure 4. Exposed floor of cranial fossa

Fixation and Processing

Immediately following removal, the hypophyses were placed in Bouin's fluid at room temperature and left to fix for 12 hours. They were then transferred to 30% ethanol and sent to the laboratory at Potchefstroom University for Christian Higher Education where they were dehydrated in ethanol and embedded in paraffin wax [43]. Sections (5 μm thick) were cut and floated onto slides pretreated with poly-L-lysine [23, 32]. These were stained using immunocytochemical techniques.

Immunocytochemistry

The paraffin sections were dewaxed and the endogenous peroxidase in the tissue blocked by treating the sections with 0,3 % hydrogen peroxide in methanol for 30 minutes [7]. Sections were then hydrated through a series of ethanol solutions of decreasing concentrations and transferred to 0,05 M Tris-saline. Nonspecific staining was reduced by incubating the sections with 10% pure swine serum which blocks the sites responsible for the non-specific staining. Both the indirect peroxidase and the peroxidase-anti-peroxidase methods [55, 54] were used to demonstrate hypophyseal endocrine cells.

Indirect peroxidase method

The sections were incubated with an unlabelled primary antiserum, which is an antiserum raised to the peptide to be tested, for example prolactin, somatotropin or ACTH, allowing the peptide and antiserum to interact. After washing a secondary antiserum, raised to react against the immunoglobulin of the species utilised for the production of the primary antiserum and labelled with horseradish-peroxidase, was applied. The secondary antiserum reacts with the primary antibody and can be visualized by the histochemical procedure of Graham and Karnovsky (1966).

Peroxidase-anti-peroxidase method

First the sections were incubated with an unlabelled primary antiserum, as described above in the indirect peroxidase method. After washing the sections they were incubated with an excess of secondary antiserum, the same utilised in the indirect peroxidase method, raised against the immunoglobulin of the species providing the first antiserum. Since it is used in excess, the secondary antiserum will still have available binding sites. These sites are utilised to react to a stable peroxidase-anti-peroxidase complex, which serves as a third layer. These reaction sites are then demonstrated using the method described by Graham and Karnovsky (1966).

Demonstration of reaction sites

In the method described by Graham and Karnovsky (1966), reaction sites are demonstrated by adding 3,3'-diaminobenzidine, which serves as an electron donor to the horseradish-peroxidase or peroxidase-anti-peroxidase complex, in the reaction. The subsequent redox reaction polymerizes the 3,3'-diaminobenzidine to a brown insoluble substance which is permanent and can be seen by light microscopy.

Primary antisera

Primary antisera supplied by Professor J.M. Polak[c] were:

- # Prolactin (Code 556)
- # Somatotropin (Code 134)
- # ACTH (Code 133)

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[c] Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, London

Optimal staining

Sections were treated with a range of dilutions of the relevant primary antisera. These sections were treated with the indirect peroxidase and peroxidase-anti-peroxidase methods and viewed with a light microscope. The dilution resulting in the best contrast between immunostained cells and the background was chosen as the optimal dilution for staining for that specific peptide.

Controls for Immunocytochemistry

The diluted primary antiserum was pre-absorbed by 20 µg/ml of the peptide it was raised against so as to bind to it. Alternatively the primary antiserum was replaced by a non-immune serum which should not bind to the relevant hormone reaction sites. As reaction sites are either completely free or only partially occupied the immune complexes in either the IP and PAP methods will not be able to react with them resulting in either reduced or abolished staining. Sections were thus examined after treatment by the IP or PAP methods to determine that the immunostaining was either reduced or abolished.

Computer assisted image analysis

Ten sections (150 000 μm^2 /section) from each animal were examined microscopically. All immunoreactive images were counted and the surface area of every image measured by means of computer assisted image analysis using the Cambridge Instruments Quantimet 520 System Version V04.02 July 1989. This system employs a video camera connected to a microscope to relay the microscopic field to the system and a computer monitor. After calibration of the system to allow for the correct transmitting factor used, a so called blank field was set up where a blank microscopic slide was inserted into the microscope and with the "Shading" function the blank field was set to "ON". A slide with a section mounted on it was then inserted into the microscope and

the image was set up, utilising the "Gain" and "Offset" functions to adjust the grey and background shading of the images, so as to obtain a clear image with adequate contrast between the background and the images. The "Measurement frame", portrayed as a blue frame inside the red detection frame, was then set so as to measure an area of 150 000 μm^2 (Figure 5). The immunoreactive images, stained brown by the immunocytochemistry procedure and thus portrayed as black images (Figure 6), were then detected by adjusting the white and black thresholds of the system so as to get adequate detection . These detected images were then amended so as to fill the gaps and saved as Bitplane 1(red) (Figure 7). With the "Feature accept" function of the system the lower and upper levels were set so as to eliminate small particles and very large confluent areas. The images were then measured, counted and saved as Bitplane 2 (green) so as to allow the operator to control the images selected (Figure 8). The total immunoreactive images counted in a field and the surface area (μm^2) of each immunoreactive image, including images that comprise of more than one cell, were recorded on a computer printout.

Statistics

All data from the computer printout were fed into the SPSS for MS WINDOWS Release 6.1 statistical analysis software package. The total immunoreactive images counted, the mean immunoreactive image surface area as well as the proportion of total area stained for each individual animal were calculated. Data not normally distributed were subjected to an appropriate transformation before statistical analysis to determine the significance of differences for total immunoreactive images, mean immunoreactive image surface area and proportion of total area stained within and between the various groups.

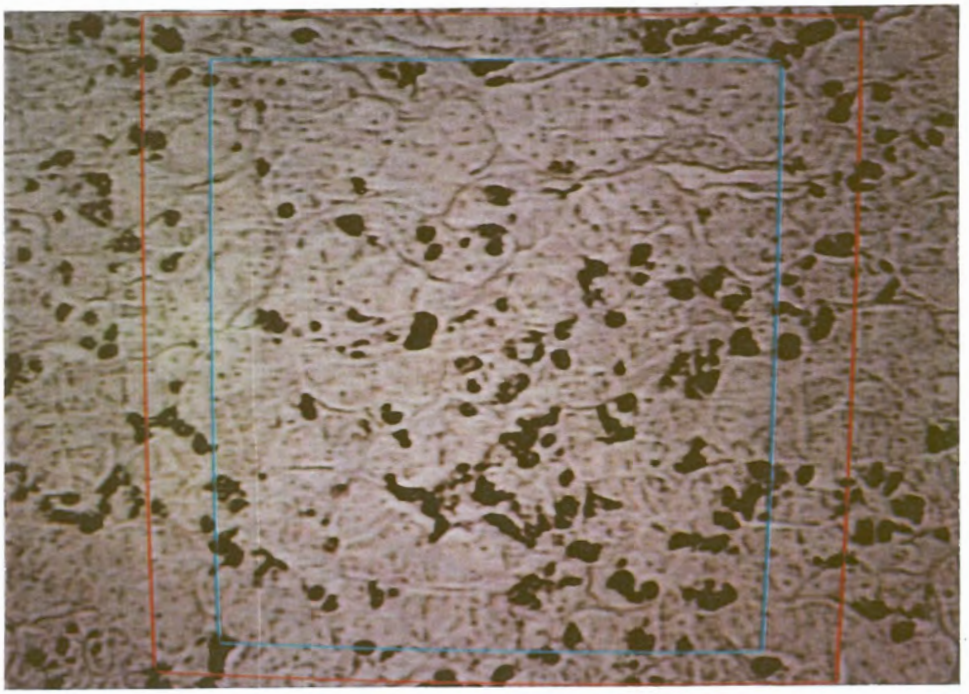

Figure 5. The "Measurement frame' (150 000 μm²), portrayed as a blue frame, inside the "Detection frame", portrayed as a red frame

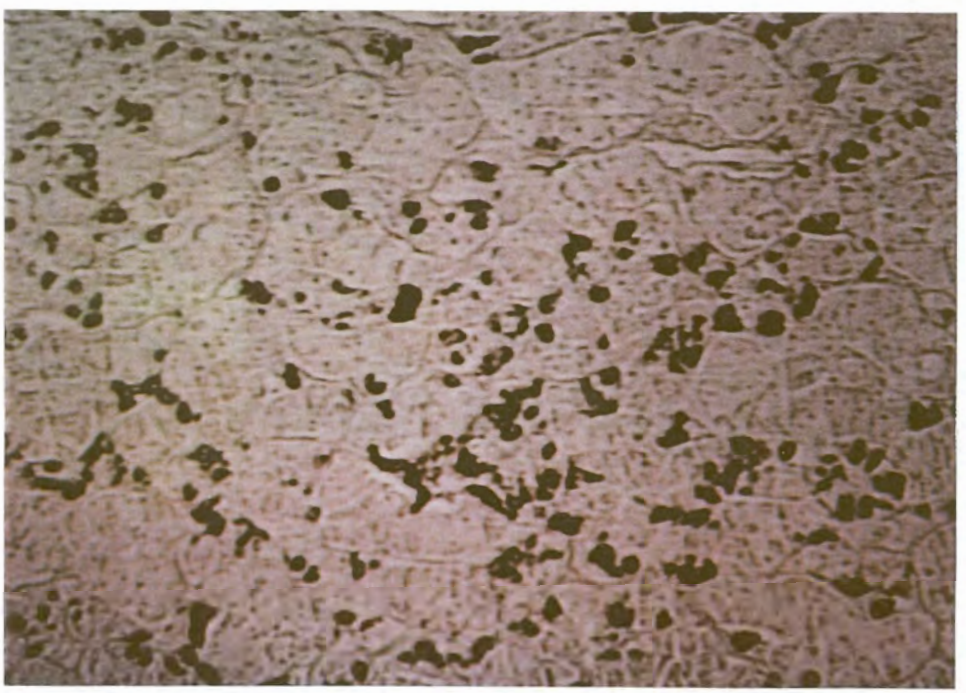

Figure 6. Microscopic field of somatostatin immunoreactive areas of a juvenile male impala as relayed to the computer monitor of the Cambridge Instruments Quantimet 520 System. IP x 200 as projected on the monitor

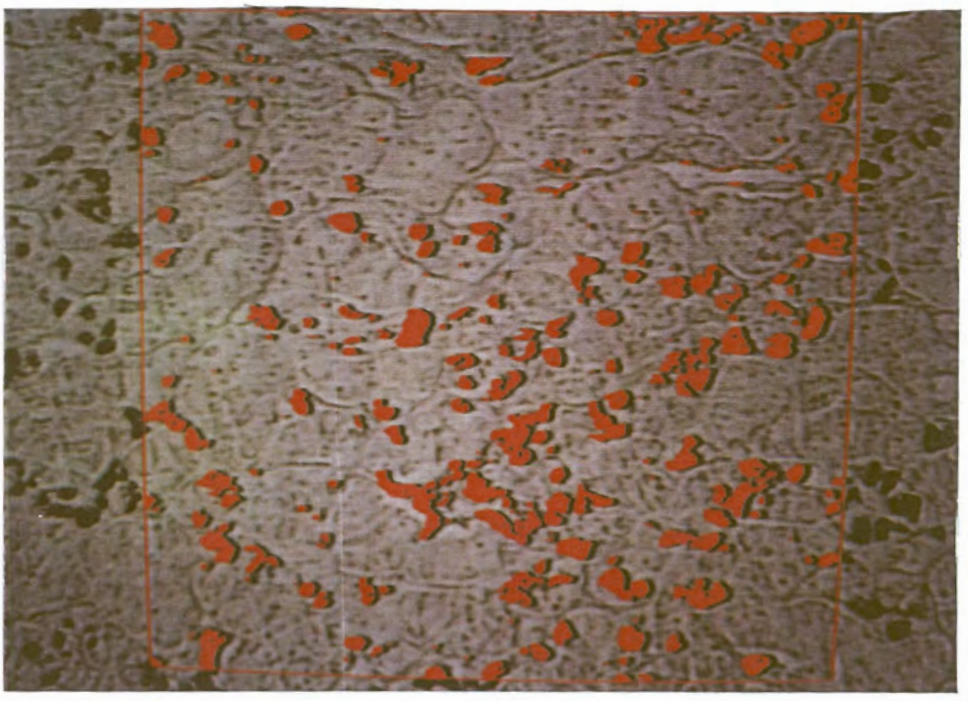

Figure 7. Detected somatostatin immunoreactive images (red) of a juvenile male impala as amended and saved as Bitplane 1. IP x 200 as projected on the monitor

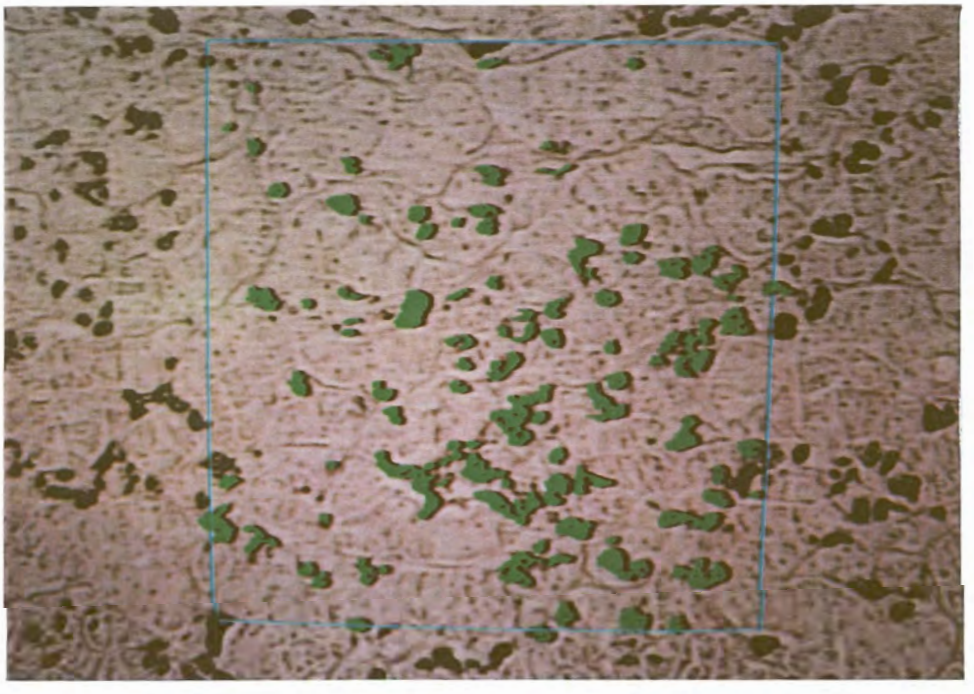

Figure 8. Measured and counted somatostatin immunoreactive images (green) of a juvenile impala ram saved as Bitplane 2. IP x 200 as projected on the monitor

The following statistical techniques were used to analyse the data:

One way analysis of variance which is used for testing of differences in the means of several groups, where the groups are defined on one independent variable only.

The Scheffe test which is used for making comparisons between two or more individual means subsequent to an Anova. The Scheffe test for multiple comparisons indicates which groups differ significantly from one another.

The T test is used for testing the difference between the means of two independent groups.

RESULTS

Prolactin

The prolactin antiserum was used at a final dilution of 1:2000. Due to the positive skew distribution of the data from the computer printout, it was transformed by log10 to fall inside the acceptable limits for kurtosis and skewness.

Table 1. Summary of the total prolactin immunoreactive images (PII) counted, Mean prolactin immunoreactive image surface area after transformation (Mean PIIA), Standard Deviation and Proportion of total area stained (PTAS) for the non-lactating and lactating groups

Group	Total PII	Mean PIIA (μm^2)	Standard Deviation	PTAS
Non-lactating (n=10)	1090	3,0467	0,3680	0,01277
Lactating (n=10)	1202	3,0751	0,3680	0,01418

Significant differences for the total immunoreactive images, at 5% level, and the mean prolactin immunoreactive image surface area ($p < 0.05$, 5% level, 2-tail) were established between individuals within each group.

Further analysis of the two groups using the parametric t-test showed a significant increase, at the 5% level, of the total immunoreactive images when lactating ewes were compared with non-lactating ewes, as well as a significant increase of the mean immunoreactive image surface area of lactating ewes when compared to non-lactating ewes ($p < 0.05$, 5% level, 2-tail). However, no

significant difference could be established between the two groups for the proportion of total area stained.

Furthermore, the Pearson product moment ($r = 0.03$) showed that the differences might not be important for all practical reasons, thus no significant conclusions from these differences should be drawn.

Data for the individuals is presented in the Appendix, Table 1.

Somatotropin

The somatotropin antiserum was used at a final dilution of 1:2000. As the data fell within the parameters for kurtosis and skewness it was not necessary to transform it.

Table 2. Summary of the total somatotropin immunoreactive images (SMTII) counted, Mean somatotropin immunoreactive image surface area (Mean SMTIA), Standard Deviation and Proportion of total area stained (PTAS) for the one year old and adult male groups

Group	Total SMTII	Mean SMTIA (μm^2)	Standard Deviation	PTAS
Juvenile males (n=9)	2927	855,82	1192,40	0,1856
Adult males (n=7)	1627	563,72	633,91	0,0874

Significant differences for the total immunoreactive images, at 1% level, counted per individual, and for the mean somatotropin immunoreactive image surface area ($p < 0.05$, 1% level, Kruskal Wallis 1-way Anova and Scheffe procedure) were established between individuals within each

group.

Further analysis of the two groups showed a significant increase, at the 1% level, of the total immunoreactive images counted for juvenile rams when compared to adult impala rams, a significant increase of the mean somatotropin immunoreactive image surface area of juvenile rams when compared with adult impala rams ($p < 0.05$, 1% level, 2-tail) as well as a significant increase, at the 1% level, in the proportion of total area stained for juvenile rams.

Data for individuals is presented in the Appendix, Table 2.

ACTH

The ACTH antiserum was used at a final dilution of 1:8000. Due to the positive skew distribution of the data, this was transformed by log10 to fall inside the acceptable limits for kurtosis and skewness.

The Scheffe test showed significant differences for the total immunoreactive images counted per individual, between individuals within five of the groups, especially in the lactating, juvenile, adult and enclosure (21 days) groups, for the mean ACTH immunoreactive image surface area (5% level) as well as for the proportion of total areas stained. However, the enclosure (7 days) and the non-lactating groups showed no significant differences between the individuals.

The lactating, juvenile and the enclosure (day 1) groups mean ACTH immunoreactive image surface area were significantly larger when compared to the other seven groups with the Scheffe test (5% level). A significant difference (1% level) between the total immunoreactive images counted for each group was also established with the lactating and juvenile groups significantly

more, and the adult and enclosure (1 day) groups significantly less than the average.

Table 3. Summary for the groups for Total ACTH immunoreactive images (ACTHII) counted, Mean ACTH immunoreactive image surface area after transformation (Mean ACTHIA), Standard Deviation and Proportion of total area stained (PTAS)

Group	Total ACTHII	Mean ACTHIA (μm^2)	Standard Deviation	PTAS
Non-lactating (n=10)	2504	2,1327	0,1514	0,02413
Lactating (n=10)	3252	2,1401	0,1522	0,03188
Juvenile (n=9)	3113	2,1363	0,1542	0,03369
Adult (n=7)	1659	2,1172	0,1535	0,02210
Enclosure (1 Day) (n=7)	1675	2,1414	0,1547	0,02358
Enclosure (7 Days) (n=8)	2311	2,1215	0,1544	0,02721
Enclosure (21 Days) (n=8)	2343	2,1209	0,1533	0,02754

The proportion of total area stained, at 5% level, was significantly more for the juvenile group when compared to the lactating, non-lactating, adult and enclosure (day 1) groups. It was also significantly more, at the 5% level, for the lactating group when compared to the non-lactating, adult and enclosure (day 1) groups.

Data for the individuals are presented in the Appendix, Table 3.

DISCUSSION

Prolactin

Prolactin, a known lactogenic peptide hormone secreted by lactotrophs in the anterior hypophysis [19], is responsible for the development of the mammary glands as well as milk production [37, 61]. These cells are active during lactation when they become both hyperplastic and hypertrophic [19]. During this phase, the secretory granules increase in size. Halmi (1983) recorded an example in which the size of each secretory granule was less than 200 nm in diameter in the resting phase, but which increased to about 600 nm in diameter during the active phase. He did not, however, mention the species of mammal in which this was recorded.

The mean prolactin immunoreactive image surface area recorded by computer assisted image analysis was greater in lactating than non-lactating ewes, rejecting the null-hypothesis of no measurable difference in the prolactin immunoreactive image surface areas of the two groups. The effect size, as calculated by the Pearson product moment, supported by the no significance difference in the proportion of total area stained, suggests that the measurements cannot provide reliable information. This may be the result of a sampling error, because ewes with milk in their udders were regarded as lactating ewes, but may already have weaned their young. This may have been compounded by the swiftness by which weaning occurs in impalas [51] and the dramatic effect weaning has on the lowering of the level of circulating prolactin [48]. It may have been further exacerbated by external factors such as stress [44, 49, 4, 36, 58, 3, 26] temperature [10, 4, 39], exercise and particular limitations of computer assisted image analysis, where it is often impossible to distinguish individual prolactin immunoreactive images. Large confluent prolactin immunoreactive images were often visualised and these contributed to the positive skewness of

the data (Figures 9 & 10).

The significant increase in the total prolactin immunoreactive images recorded can be explained either by hyperplasia or hypertrophy of lactotrophs, trans-differentiation of somatotrophs to lactotrophs or a combination of the above [7, 53, 45].

Nevertheless, this study has shown that immunocytochemistry in combination with computer assisted image analysis is sensitive and able to discriminate between lactating and non-lactating impala ewes on the basis of the total immunoreactive images and mean prolactin immunoreactive image surface area in the hypophysis.

Somatotropin

Somatotropin, a peptide hormone structurally very similar to prolactin [37, 33], is secreted by specific acidophils, somatotrophs, which are mainly found in the posteriolateral part of the pars distalis of the anterior hypophysis [19, 1, 45]. In a study of the acidophyllic cells of Egyptian buffalo foeti somatotrophs appeared to be the first to differentiate, as early as the second month of gestation [52]. They are numerous, small rounded cells, fairly uniform in size, with granules ranging in diameter from 300 nm to 370 nm [19, 43], occupying approximately a third of the pars distalis [1]. During lactation, somatotrophs are believed to be recruited to produce prolactin [53].

Somatotropin is responsible for growth in the young animal by increasing chondrogenesis, protein synthesis and other metabolic functions [10]. Absence of this hormone leads to dwarfism, and over secretion to gigantism in the juvenile and acromegaly in the adult [19].

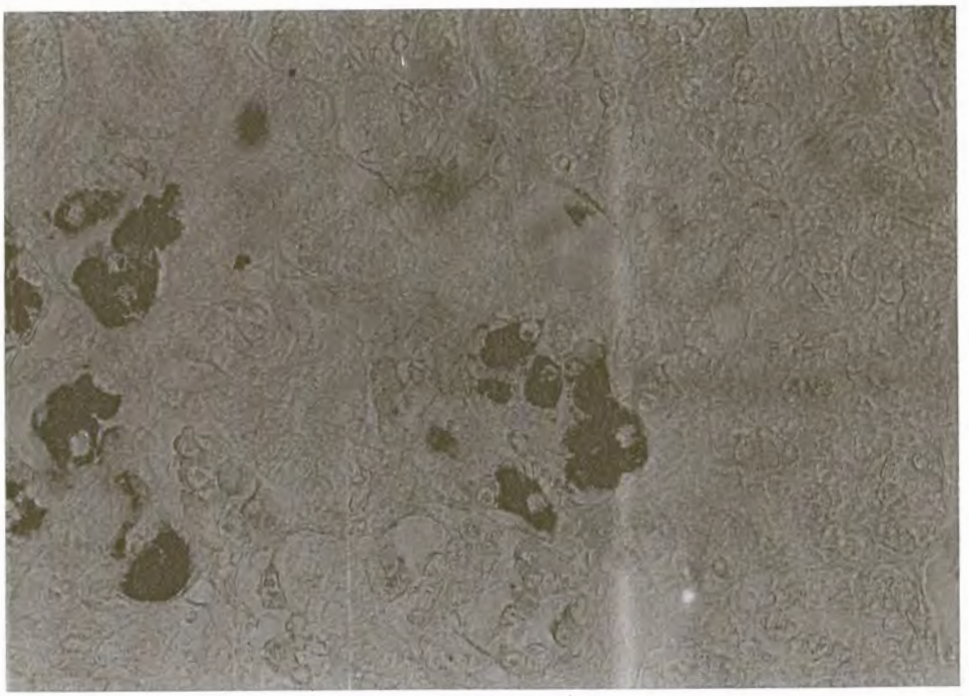

Figure 9. Individual prolactin immunoreactive areas of a non-lactating impala female. PAP x 1200

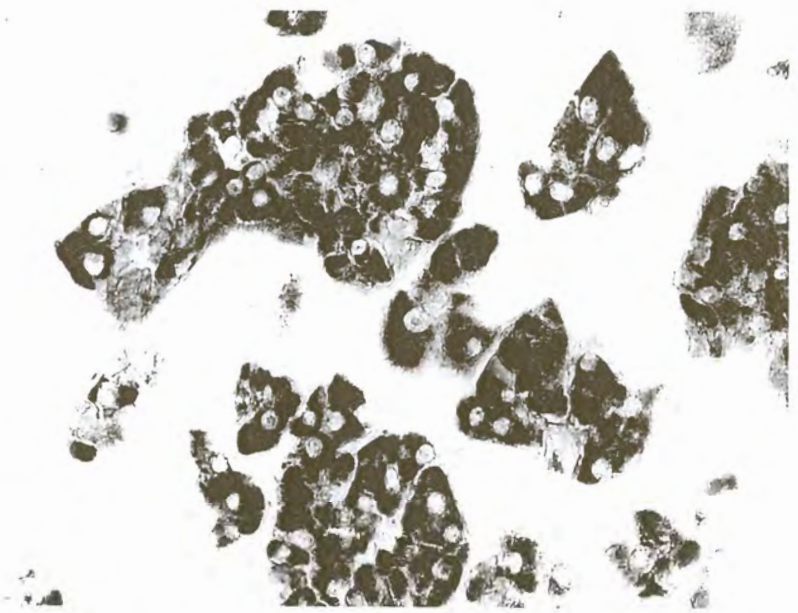

Figure 10. Large confluent prolactin immunoreactive areas in a lactating impala female. PAP x 1200

The mean somatotropin immunoreactive image surface area recorded by computer assisted image analysis was significantly greater in juvenile than adult impala rams. Furthermore, the total immunoreactive images counted were also significantly higher in the juvenile group compared to the adult group. These differences are further supported by the significant larger proportion of total area stained for juveniles.

As somatotropin is a hormone responsible mainly for growth, these results confirm that juvenile animals have not only more cells active for this hormone, but that the mean immunoreactive image surface area and proportion of total area stained are also greater. Wahba (1988) showed that the surface area of acidophillic cells, believed to be somatotrophs, as measured by a micrometer increased dramatically in Egyptian buffalo foeti from the second month to the tenth month of gestation, confirming that the cell surface area is proportional to the activity of the cell. Furthermore, Schwarz (1992), demonstrated that the levels of somatotropin decreased during growth in Simmental cattle, confirming that the activity of somatotrophs decreases with age.

Significant differences were also recorded for the mean somatotropin immunoreactive image surface area between individual impala in the same group in this study. This might be the result of some external factors, proven to cause a change in circulating somatotropin, for instance sleep [38], physical exercise [38], environmental stress such as cold spells [34, 38], food deprivation [37, 6, 24, 47], psychological stress [37], trauma [37] and fever or disease [37, 57]. All the animals were not culled in the same place, at the same time of day, nor investigated for the presence of any injuries or disease. The differences might have been further confounded by limitations of computer assisted image analysis, where it was again often impossible to distinguish individual somatotropin immunoreactive images. Large confluent somatotropin immunoreactive images were often

visualised.

Nevertheless, this second study confirmed that immunocytochemistry, combined with computer assisted image analysis, is sensitive enough and able to discriminate between the hypophyses of juvenile and adult impala rams. It was therefore accepted that the combination of these techniques could be used in the follow up study to discriminate between the ACTH immunoreactive images of various groups of animals in various states of stress.

ACTH

ACTH, a polypeptide hormone, is secreted by corticotropes, distributed evenly within the anterior lobe of the hypophysis [37, 42]. It controls the size, structure and vascularity of the adrenal cortex, thereby the production and release of corticoids, especially cortisol in man [37]. The secretion of ACTH is controlled by corticotropin releasing hormone, secreted by the hypothalamus, and cortisol via negative feedback [37]. Stress, fear, capture, adrenalin and other stimuli cause an increase in the secretion of ACTH, either directly by the effect of epinephrine on the hypophysis, or indirectly via hypothalamic stimulation through secretion of corticotropin releasing hormone which stimulates the hypophysis to secrete ACTH [37, 26, 52, 50]. Continual stimulation of the hypophysis is necessary to ensure the survival of an animal.

As was the case with prolactin and somatotropin, there were significant differences in the mean ACTH immunoreactive image surface area and total immunoreactive images amongst five of the different treatment groups of impala. Two of the seven groups showed no significant differences within the group. This might again be the result of limitations of computer assisted image analysis, where it was again often impossible to distinguish individual ACTH immunoreactive

images. Large confluent ACTH immunoreactive images were often visualised. However, individual animals have differing abilities to cope with stress. For instance, the corticotropes cell size of pigs chosen to produce so-called low-fat meat were 70 % larger than those producing high-fat meat [42]. Stress susceptible pigs were also shown to have significantly higher resting ACTH levels [35]. An increase in the cross sectional cell size of hypophyseal corticotropes due to stress was also demonstrated in pigs [42]. Continual interaction between the animal and its changing environment appears to be essential in maintaining optimal corticosteroid production. It has been reported for example that some wild animals kept in zoos develop partial hypophyseal atrophy as a result of prolonged inactivity. This rendered the animals more susceptible to shock with resultant deaths [8].

When the groups were compared to each other the mean ACTH immunoreactive image surface areas were significantly higher in the lactating, juvenile and enclosure (1 day) groups. When comparing ACTH immunocytochemical reactivity of the seven groups of impala for the total immunoreactive images counted, the lactating and juvenile groups, showed significant differences above the average immunoreactive images counted, and two groups, the adult and enclosure (1 day) groups, showed significant differences below the average immunoreactive images counted. The significant differences for the mean ACTH immunoreactive image surface area and total immunoreactive images counted for the lactating and juvenile groups were further supported by the significant increase of the proportion of total area stained when these two groups were compared to the other groups.

Lactation, is a stressful event and it could thus be expected that this group's corticotropes would have been more active on a continuous basis during the period of lactation than in the other

animals. This is reflected in the increased secretion of glucocorticoids in order to meet the extra energy requirements for lactation. This was further supported by the fact that the total ACTH immunoreactive images counted and the proportion of total area stained in these impala were also significantly higher than the averages. Stress was shown to lead to an increase in the size of corticotropes in pigs [42].

Juvenile male animals are in constant threat, not only from adult rams displaying their dominant behaviour, but also from predators and fellow impalas in competition for food, causing considerable environmental stress. This could have led to the significant increase in the mean ACTH immunoreactive image surface area, the significant increase in the total immunoreactive images counted and the significant larger proportion of total area stained.

The enclosure (1 day) group showed a significant increase in the mean ACTH immunoreactive image surface area, but, showed a significant decrease in the total immunoreactive images counted as well as a significant smaller proportion of total area stained if compared with the juvenile group. As stress causes an increase in the secretion of ACTH [37, 52] the significant increase in the mean ACTH immunoreactive image surface area could be explained. However, the reason for the significant decreases in the total immunoreactive images counted and the proportion of total area stained are not clear. It might, however, be due to the depletion of the hormone within the corticotropes as the animals experienced acute stress over a 1 day period with a lag period in the response of the corticotropes to react to the continuous stressors. This is supported by the fact that not only the mean ACTH immunoreactive image surface area, but also the total immunoreactive images slowly normalised over time as no significant differences could be shown between the enclosure (7 days), enclosure (21 days) and the other groups.

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i. 14748319

Animals adapt to chronic stress, such as that experienced by the groups kept captive in the enclosure for a period exceeding 1 day, or in the case of free-ranging adult animals under continual environmental stress. This is evidenced by the lack of significant changes and even decreased secretion of circulating ACTH levels as demonstrated in experimental animals [26, 20, 50, 11]. It appears that under conditions of chronic stress a change in the functioning of the hypophyseal-adrenal axis occurs altering the ability of the hypothalamic-hypophyseal-adrenal axis to respond to a novel stimulus [7]. This is probably manifested by an enhanced responsiveness of the adrenals to ACTH, giving rise to significantly increased corticoid and cortisol secretion. This could be reflected as a minimal increase in ACTH secretion by the hypophysis, when the animal is exposed to a novel stressor [25, 2]. This adaptation, might be the reason for the adult group showing significantly lower total images counted, but no significant difference from the other groups for the mean ACTH immunoreactive image surface area and the proportion of total area stained. Depletion of the corticotropes resulting in non-staining of the cells, however, may also be a factor for the significantly lower total images counted, but no evidence supporting this argument could be found in literature.

As with the studies on prolactin and somatotropin, it was found that the combination of these two techniques was sensitive and able to discriminate between animals exposed to acute stress and animals under less stressful conditions. However, probably due to the adaptation of animals to chronic stress, these techniques were not sensitive and able to discriminate between animals under normal conditions and animals exposed to chronic stress, such as the enclosure (7 and 21 days) groups. Some other technique, such as ACTH challenging, successfully employed in the determination of the best practise in the housing of hens [31] and in restraining, weaning and transport of calves [19], could perhaps be employed in order to discriminate between these groups.

Cortisol, which is secreted due to a stimulus from ACTH, may in some cases be a better measure of stress, as it is more responsive, be it under acute stress or chronic stress situations. The cortisol responsiveness appears to be enhanced in situations of chronic stress [2, 46, 20, 41, 4, 25].

CONCLUSION

Stress is of paramount importance in the management of wildlife populations and may result in significant losses due to mortalities [30]. Acute stress in the course of wildlife management has been intensively investigated [3, 14, 22, 16, 15, 20, 8, 28, 29, 60]. Chronic stress, on the contrary, has only been researched in domesticated animals and very few references pertaining to wildlife could be found[1]. This is probably due to the difficulty in measuring chronic stress as the response to the restraint needed to examine the animal or collect blood samples overrides the responses to chronic stress.

Although many factors influence the secretion of hypophyseal hormones, one of them is stress [44, 46, 26, 42, 50, 6, 2, 11]. Chronic stress, however, seems to lead to adaptation within individuals at the hypophyseal level [7, 50, 11] and manifests itself in an alteration in the ability of the hypothalamus-hypophyseal-adrenal axis to respond to a novel stressor [7, 2]. This probably results in the enhanced adrenal responsiveness to secrete corticoids, especially cortisol, following stimulation by ACTH . This adaptation may nullify attempts to develop a measurable parameter for chronic stress by the use of the combination of immunocytochemistry with computer assisted image analysis to measure changes in ACTH secretions. The technique was found to be very useful in discriminating between diverse groups, such as juvenile and adult animals. It would, however, be of interest to compare a group of impala from one of the military terrains with the state of adaptation of similar animals in the Kruger National Park, using these methods.