IMMUNOCYTOCHEMISTRY, ASSISTED BY COMPUTER IMAGE ANALYSIS, OF HYPOPHYSEAL PEPTIDE HORMONES OF THE IMPALA *(AEPYCEROS MELAMPUS)*

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RESUME

The South African National Defence Force has vast areas of land under its control, often occupied by wildlife, which it uses as training ground for its forces. As the extent to which military activities influences the environment is not commonly known, the SANDF has embarked on research programs to investigate the effects of these activities on the resident wildlife populations in order to manage them optimally.

The measurement of plasma hormone concentrations using radioimmunoassays is commonly used in the evaluation of stress in domestic animals. Such assays are costly, require a degree of sophistication in using specialised techniques in the veld and are difficult to interpret as the stress of restraining wild animals results in acute stress responses. It was thus decided to evaluate the use of immunocytochemistry, a more stable method, combined with computer image analysis, not only to visualise hypophysial cells active for hormones, but also to attempt to determine the magnitude of suspected structural changes. As the combination of these techniques would be utilised for the first time it was decided to first evaluate the technique under known differing physiological circumstances, for example in lactating and in growing animals. If this was successful it would be evaluated to estimate changes in ACTH secretion under different stress circumstances, for example free ranging animals and animals kept in an enclosure for various periods of time.

Impalas (*Aepyceros melampus*) are abundant and thus easily available for the study of their physiology. Animals used in this investigation were culled as part of other projects undertaken in the Kruger National Park.

Lactating impala ewes demonstrated significantly more prolactin immunoreactive images and an increased mean immunoreactive image surface area ($p < 0.05$, 5% level, 2-tail) compared to non-lactating ewes rejecting the null-hypophyses of no difference. However, no significant difference could be demonstrated for the proportion of area stained between the two groups.

Significantly more somatotropin immunoreactive images, an increased mean immunoreactive image surface area ($p<0.05$, 1% level, 2-tail) as well as a significant larger proportion of total area stained were found in juvenile one year old male impala rams compared to those adult males. This supported the evidence of the prolactin trial, that the combination of these techniques is sensitive and able to distinguish between two physiologically different groups.

Lactating impala, juvenile animals and animals that had been kept in an enclosure for 1 day demonstrated significantly increased mean ACTH immunoreactive image surface area (5% level, Scheffe test) when compared to non-lactating, adult or animals that were kept in an enclosure for 7 and 21 days respectively. The lactating and juvenile animals demonstrated significantly more, and the adult and enclosure (1 day) groups significantly less total ACTH immunoreactive images when compared. The juvenile group also demonstrated a significant larger proportion of total area stained than the lactating, non-lactating, adult and enclosure (day 1) groups, and the lactating group a significant larger proportion of total area stained than the non-lactating, adult and enclosure (day 1) groups.

The combination of immunocytochemistry and computer assisted image analysis proved to be useful in discriminating between physiologically diverse groups. It was, however, not successful in discriminating amongst chronic stressed and free ranging animals.

SAMEVATTING

Die Suid Afrikaanse Nasionale Weermag het groot gebiede grond onder sy beheer waar wild dikwels vrylik op voorkom. Omrede die effek van militêre bedrywighede op die natuur nie alombekend is nie, het die weermag navorsings programme van stapel gestuur om die effekte te probeer bepaal ten einde die terreine optimaal te bestuur.

Die meting van plasma hormoon konsentrasies dmv radioimmuno opnames word algemeen gebruik om stres te evalueer in huisdiere. Sulke opnames is duur, vereis 'n graad van sofistikasie in die gebruik van gevorderde tegnieke in die veld en is moeilik om te interpreteer omrede wilde diere wat gevang moet word akute stres response ondergaan. Om die bogenoemde faktore sover moontlik te beperk is besluit om immunositochemie, 'n meer stabiele metode, in samewerking met rekenaar gesteunde voorwerp analise te gebruik om die hipofiseale selle aktief vir hormone, sowel as die moontlike omvang van die strukturele verandering van die selle, te bestudeer. Omrede die kombinasie van die tegnieke nog nie voorheen gebruik is nie is daar besluit om dit eers op fisiologiese diverse groepe, byvoorbeeld lakterende diere en jong groeiende diere, te toets, en indien suksesvol, dit dan aan te wend om adrenokortikotropien hormoon te evalueer onder verskeie stres toestande, byvoorbeeld vry lewende diere en diere wat in bomas aangehou word vir verskeie tydperke.

Rooibokke (*Aepyceros melampus*) kom algemeen voor en dus geredelik beskikbaar vir studies aangaande hul fisiologie. Diere gebruik in hierdie studie is geskiet as deel van ander navorsingsprojekte in die Nasionale Kruger Wildtuin.

Lakterende rooibok ooie het beduidend meer prolaktien immunoreaktiewe voorwerpe en 'n beduidend groter gemiddelde immunoreaktiewe voorwerp oppervlakte (p<0.5, 5% vlak, 2 -tail) vergeleke met nie-lakterende ooie. Dit verwerp dus die nul-hipotese van geen verskil tussen die groepe. Daar kon egter geen beduidende verskil verkry word vir die proporsie van die totale immunoreaktiewe oppervlakte tussen die twee groepe nie.

Jong groeiende rooibok ramme het beduidend meer somatotropien immunoreaktiewe voorwerpe, 'n beduidend groter gemiddelde immunoreaktiewe voorwerp oppervlakte (p<0.5, 1% vlak, 2-tail) sowel as 'n beduidende groter totale immunoreaktiewe oppervlakte vergeleke met volwasse ramme. Die twee evaluasies bevestig dat die kombinasie van die tegnieke die vermoë het en sensitief genoeg is om tussen diverse fisiologiese groepe te kan onderskei.

Lakterende-, jong groeiende-, en rooibokke wat aangehou was vir 1 dag in 'n boma het 'n beduidend groter gemiddelde ACTH immunoreaktiewe voorwerp oppervlakte (5% vlak, Scheffe toets) vergeleke met volwasse-, nie-lakterende-, en rooibokke aangehou in bomas vir 7 en 21 dae respektiewelik. Lakterende- en jong rooibokke het beduidend meer, en volwasse- en rooibokke aangehou vir 1 dag in 'n boma beduidend minder totale ACTH immunoreaktiewe voorwerpe wanneer vergelyk. Die jong groeiende groep het ook 'n beduidende groter totale immunoreaktiewe oppervlakte in vergeleke met die lakterende, nie-lakterende volwasse en die groep wat 1 dag in 'n boma was. Voorts het die lakterende groep weer op hul beurt 'n beduidende groter totale immunoreaktiewe oppervlakte in vergeleke met die nie-lakterende, volwasse en die groep wat 1 dag in die boma was.

Die kombinasie van immunositochemie en rekenaar gesteunde voorwerp analise is bewys as 'n

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nuttige metode om diverse groepe aan te dui. Die tegniek was egter nie suksesvol om tussen

chroniese stres groepe en vrylewende groepe te onderskei nie.

ACKNOWLEDGEMENTS

ABBREVIATIONS

A	- Adult	
ACTH	- Adrenocorticotropic hormone	
E	- Enclosure	
J	- Juvenile	
L	- Lactating	
ml	- millilitre	
NL	- Non-lactating	
PRL	- Prolactin	
SANDF	- South African National Defence Force	
SMT	- Somatotropin	
MIEM	- Military Integrated Environmental Management	

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].

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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.

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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

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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
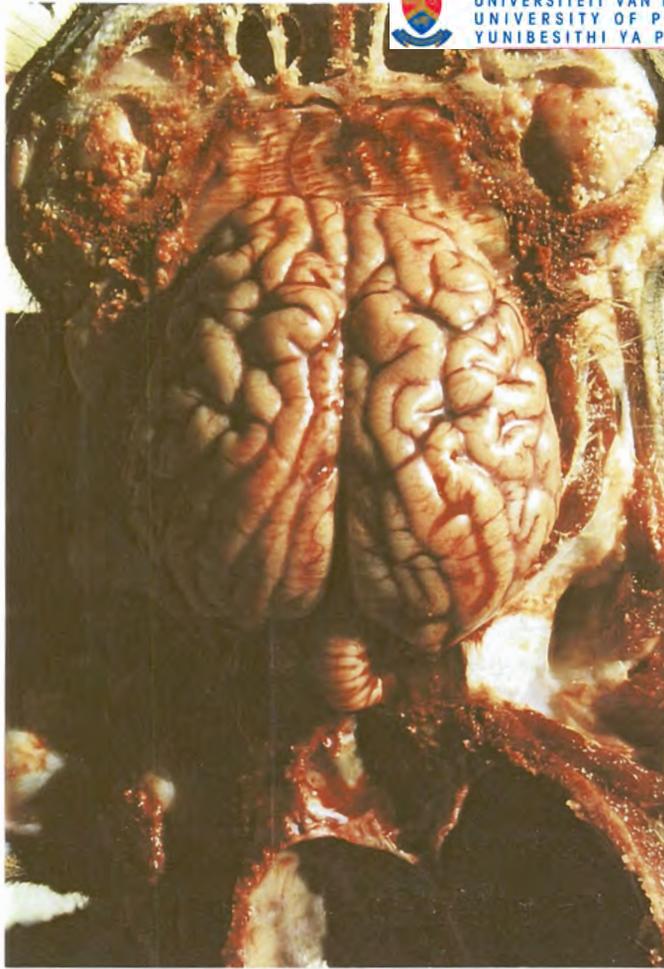
Figure 3. Bulk of the brain ready to be removed
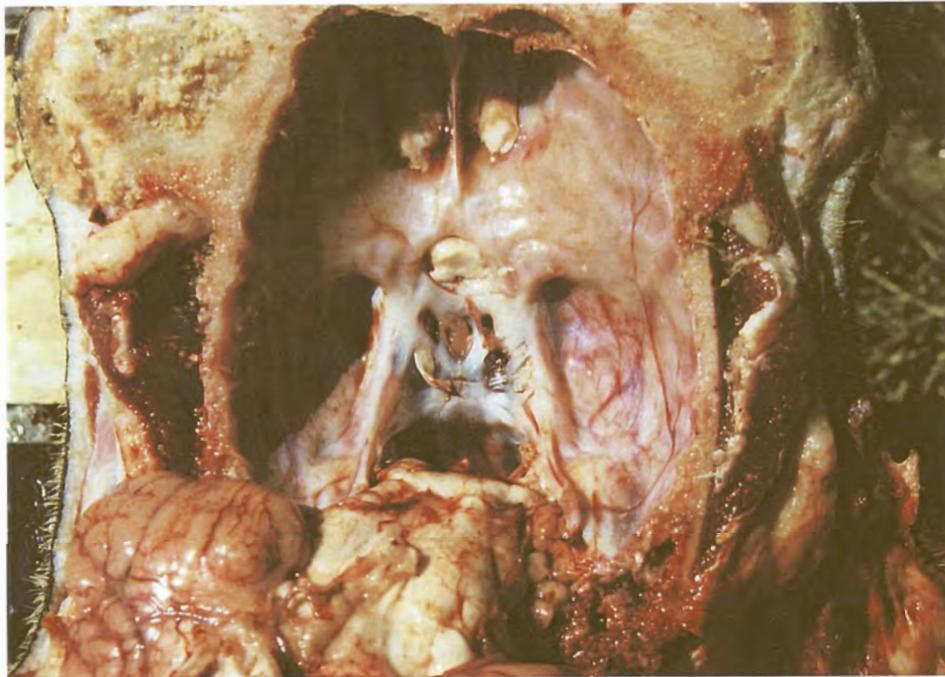
Figure 4. Exposed floor of cranial fossa

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).

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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

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<u>Optimal staining</u>

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.
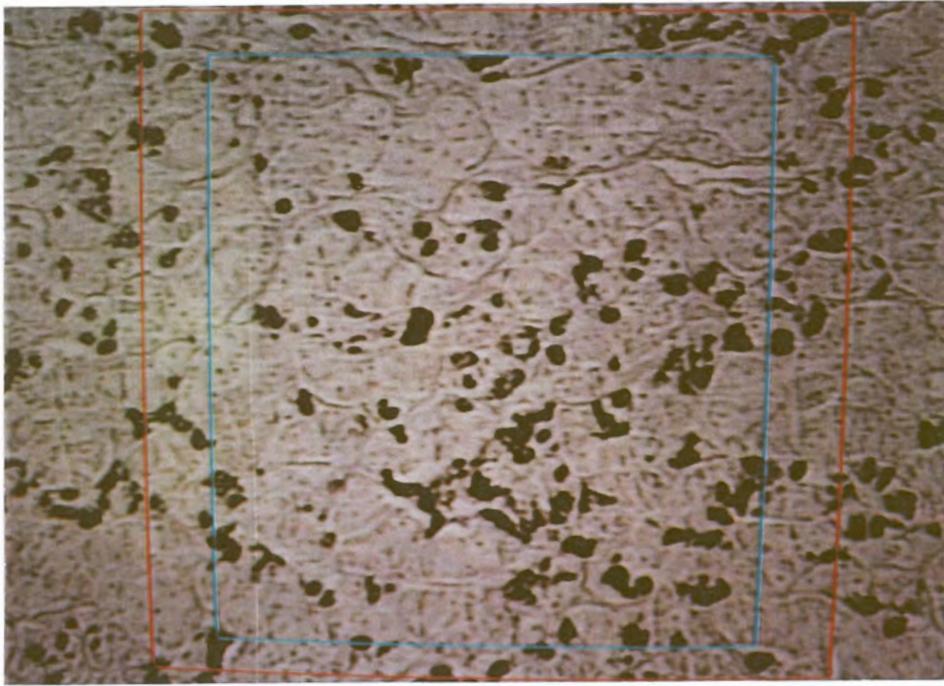
Figure 5. The "Measurement frame' (150 000 μm^2), portrayed as a blue frame, inside the "Detection frame", portrayed as a red frame
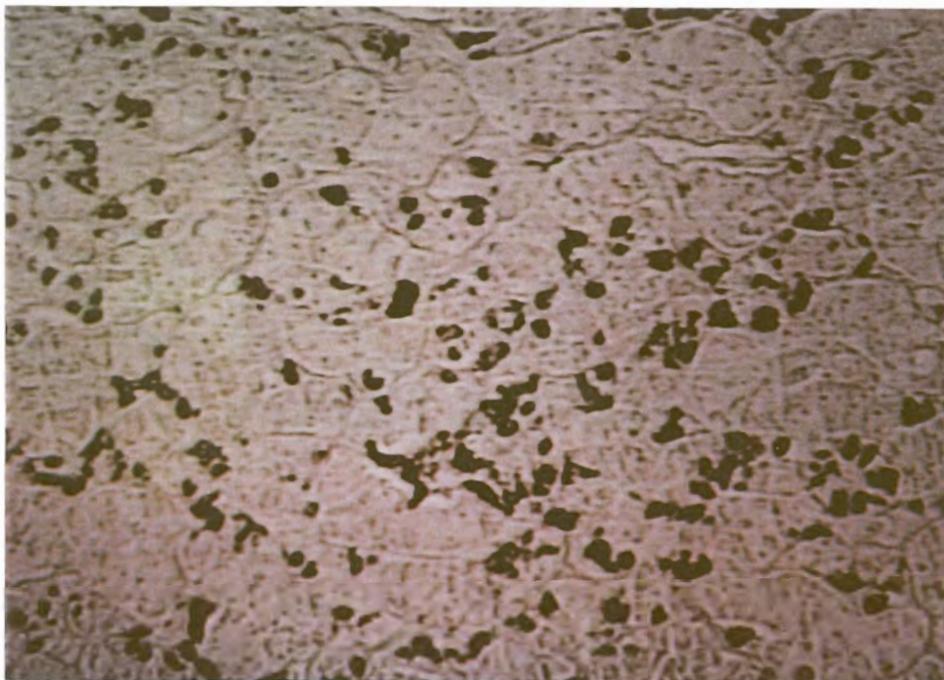
Figure 6. Microscopic field of somatostatin immunoreactive areas of a juvenile male impala as relayed to the computer monitor of the Cambridge Instruments Quantimet 520 System. IP x 200 as projected on the monitor
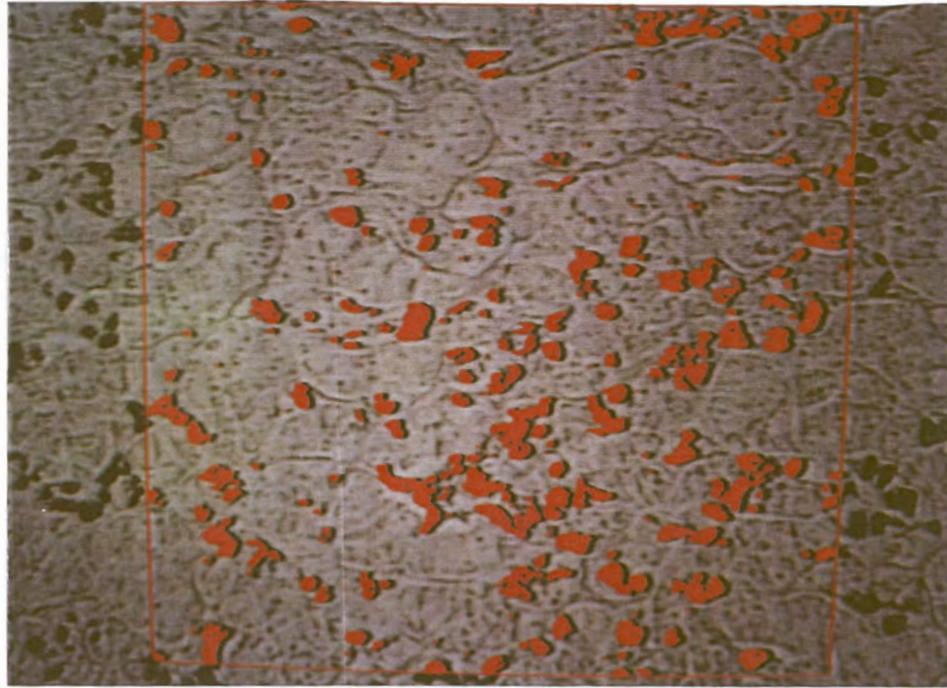
Figure 7. Detected somatostatin immunoreactive images (red) of a juvenile male impala as amended and saved as Bitplane 1. IP x 200 as projected on the monitor
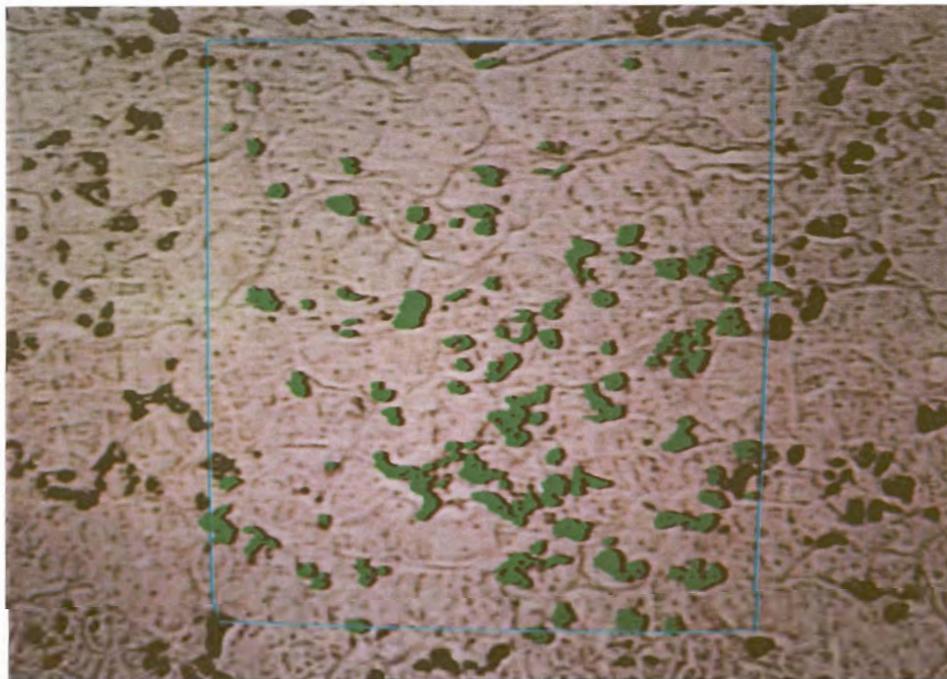
Figure 8. Measured and counted somatostatin immunoreactive images (green) of a juvenile impala ram saved as Bitplane 2. IP x 200 as projected on the monitor

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 (μm²)	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

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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

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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

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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].
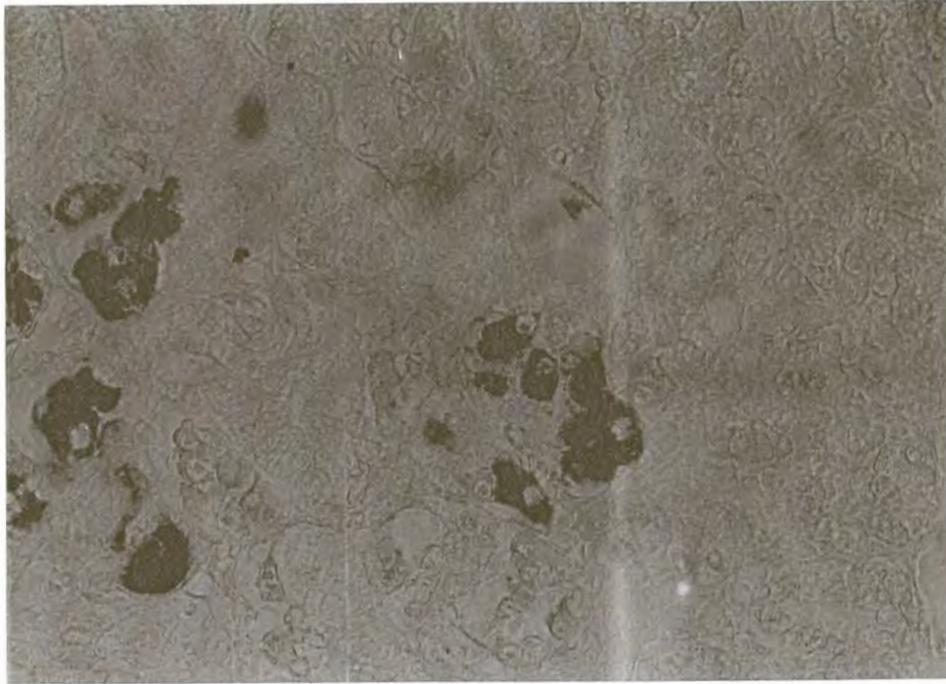
Figure 9. Individual prolactin immunoreactive areas of a non-lactating impala female. PAP x 1200
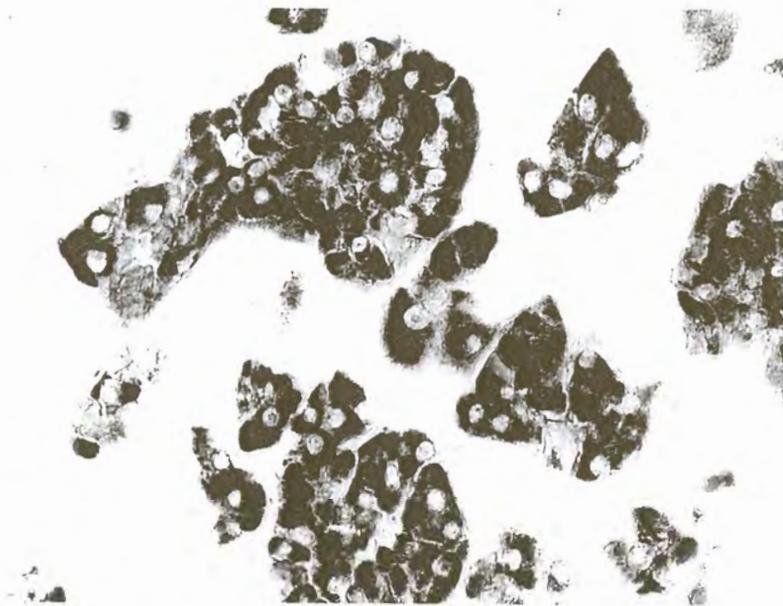
Figure 10. Large confluent prolactin immunoreactive areas in a lactating impala female. PAP x 1200

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

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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

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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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, 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.

APPENDIX

Table 1. Total prolactin immunoreactive images (PII) counted, Mean prolactin immunoreactive image surface area(MPIIA), Mean prolactin immunoreactive image surface area after transformation (MPIIAT), Standard Deviation (SD) and Proportion of total area stained (PTAS) for the non-lactating (NL) and lactating (L) groups

Animal/Group	Total PII	MPIIA (μm²)	MPIIAT	S D	PTAS
NL 1	183	161,1424	3,0500	0,3513	0,01966
NL2	108	142,7271	2,9896	0,3469	0,01028
NL3	127	167,7681	3,0370	0,3814	0,01420
NL4	134	159,4177	3,0264	0,3667	0,01424
NL5	165	158,7227	3,0308	0,3565	0,01746
NL6	117	206,1310	3,1286	0,3905	0,01608
NL7	82	132,6842	2,9716	0,3317	0,00725
NL8	56	162,7280	3,0584	0,3536	0,00608
NL9	54	252,5954	3,2038	0,4230	0,00909
NL10	64	213,0313	3,1474	0,4048	0,00909
Non-Lactating	**1090**	**175,6948**	**3,0467**	**0,3680**	**0,01277**
L1	265	158,5346	3,0304	0,3639	0,02801
L2	138	182,9749	3,0930	0,3743	0,01683
L3	44	160,4264	3,0567	0,3440	0,00471
L4	155	144,4156	3,0042	0,3367	0,01492
L5	80	146,9649	3,0211	0,3396	0,00784
L6	118	204,8796	3,1398	0,3760	0,01588
L7	73	169,1121	3,0486	0,3783	0,00823
L8	106	204,8505	3,1377	0,3856	0,01448
L9	113	211,7866	3,1649	0,3728	0,01595
L10	110	185,8706	3,1024	0,3716	0,01363
Lactating	**1202**	**176,9816**	**3,0751**	**0,3680**	**0,01418**

Table 2.   Total somatotropin immunoreactive images (SMTII) counted, Mean somatotropin

immunoreactive image surface area (MSMTIA), Standard Deviation (SD) and Proportion of

total area stained (PTAS) for the juvenile male (J) and adult male (A) groups

Animal/Group	Total SMTII	MSMTIA (μm^2)	S D	PTAS
J1	320	764,3250	1077,0379	0,1631
J2	380	632,0500	883,3562	0,1601
J3	317	487,9590	478,4198	0,1031
J4	331	849,1964	1138,8335	0,1874
J5	328	793,5427	955,3352	0,1735
J6	379	1121,9868	1526,7062	0,2835
J7	263	1048,4563	1318,5661	0,1838
J8	397	965,2317	1353,0254	0,2555
J9	212	1132,1274	1555,8380	0,1600
Juvenile	**2927**	**855,8241**	**1192,4024**	**0,1856**
A1	214	554,7804	583,9511	0,0791
A2	236	466,2415	335,5042	0,0734
A3	255	472,8824	412,3376	0,0804
A4	194	488,7062	687,4381	0,0632
A5	257	642,8405	593,2428	0,1101
A6	244	674,8770	947,9141	0,1098
A7	227	630,5595	666,4675	0,0954
Adult	**1627**	**563,7167**	**633,9105**	**0,0874**

Table 3.　　Total ACTH immunoreactive images(TACTHII) counted, Mean ACTH immunoreactive image surface area(MACTHIA), Mean ACTH immunoreactive image surface area after transformation (MACTHIAT), Standard Deviation (SD) and Proportion of total area stained (PTAS)

Animal/Group	TACTHII	MACTHIA (μm^2)	MACTHIAT	S D	PTAS
NL1	305	146,9474	2,1406	0,1496	0,02988
NL2	223	148,1042	2,1439	0,1505	0,02202
NL3	256	146,3174	2,1395	0,1474	0,02497
NL4	237	133,9093	2,1028	0,1411	0,02116
NL5	223	142,4180	2,1255	0,1524	0,02117
NL6	243	152,0364	2,1532	0,1571	0,02463
NL7	154	144,9896	2,1314	0,1596	0,01489
NL8	240	145,7887	2,1353	0,1550	0,02333
NL9	247	135,1972	2,1086	0,1364	0,02226
NL10	376	147,5416	2,1393	0,1582	0,03698
Non-lactating	**2504**	**144,5411**	**2,1327**	**0,1514**	**0,02413**
L1	250	143,5588	2,1321	0,1458	0,02393
L2	297	143,1917	2,1281	0,1525	0,02835
L3	231	137,2140	2,1094	0,1517	0,02113
L4	385	151,0205	2,1516	0,1531	0,03876
L5	313	145,9223	2,1364	0,1527	0,03045
L6	354	156,2297	2,1670	0,1522	0,03687
L7	301	156,1572	2,1672	0,1511	0,03134
L8	397	142,1178	2,1260	0,1498	0,03761
L9	415	148,9726	2,1445	0,1565	0,04122
L10	309	141,5818	2,1257	0,1460	0,02917
Lactating	**3252**	**147,0579**	**2,1401**	**0,1522**	**0,03188**

Animal/Group	TACTHII	MACTHIA (μm^2)	MACTHIAT	S D	PTAS
J1	219	160,0457	2,1773	0,1534	0,02337
J2	387	139,0992	2,1177	0,1463	0,03589
J3	396	146,3304	2,1390	0,1489	0,03863
J4	408	147,8847	2,1418	0,1544	0,04022
J5	356	140,2346	2,1195	0,1505	0,03328
J6	329	143,7588	2,1288	0,1554	0,03153
J7	319	161,7561	2,1794	0,1603	0,03440
J8	349	145,5935	2,1344	0,1555	0,03388
J9	350	137,1162	2,1086	0,1522	0,03199
Juvenile	**3113**	**146,0933**	**2,1363**	**0,1542**	**0,03369**
A1	201	132,0617	2,0959	0,1414	0,01770
A2	146	129,6295	2,0839	0,1509	0,01262
A3	266	139,6412	2,1153	0,1560	0,02476
A4	159	134,5107	2,1050	0,1418	0,01426
A5	230	154,0403	2,1566	0,1633	0,02362
A6	324	130,4483	2,0898	0,1441	0,02818
A7	333	151,3522	2,1516	0,1559	0,03360
Adult	**1659**	**139,9017**	**2,1172**	**0,1535**	**0,02210**

Animal/Group	TACTHII	MACTHIA (µm²)	MACTHIAT	S D	PTAS
E1/1	336	156,0525	2,1627	0,1623	0,03496
E1/2	373	157,4315	2,1713	0,1495	0,03915
E1/3	144	149,0677	2,1440	0,1572	0,01431
E1/4	70	137,1347	2,1100	0,1514	0,00640
E1/5	185	146,1126	2,1355	0,1572	0,01802
E1/6	290	134,6537	2,1030	0,1467	0,02603
E1/7	277	141,9683	2,1258	0,1473	0,02622
Enclosure (D 1)	**1675**	**147,8367**	**2,1414**	**0,1547**	**0,02358**
E2/1	324	138,0198	2,1120	0,1514	0,02981
E2/2	264	141,0728	2,1233	0,1478	0,02483
E2/3	253	137,1311	2,1066	0,1587	0,02313
E2/4	268	138,5315	2,1159	0,1461	0,02475
E2/5	337	149,8059	2,1442	0,1626	0,03366
E2/6	386	145,2232	2,1332	0,1562	0,03737
E2/7	188	131,6023	2,0926	0,1464	0,01649
E2/8	291	142,5605	2,1250	0,1562	0,02766
Enclosure (D 7)	**2311**	**141,3022**	**2,1215**	**0,1544**	**0,02721**
E3/1	333	150,0471	2,1440	0,1644	0,03331
E3/2	223	134,5490	2,1004	0,1519	0,02000
E3/3	248	128,7412	2,0883	0,1339	0,02129
E3/4	214	132,3134	2,0971	0,1416	0,01888
E3/5	312	132,2790	2,0951	0,1457	0,02751
E3/6	213	128,2147	2,0840	0,1399	0,01821
E3/7	233	148,8463	2,1455	0,1523	0,02312
E3/8	567	153,4129	2,1566	0,1578	0,05799
Enclosure (D 21)	**2343**	**141,0414**	**2,1209**	**0,1533**	**0,02754**

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