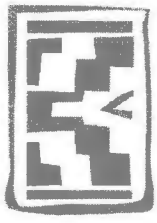

Influence of lactation on the prolactin secreting cells of the hypophysis of impala (*Aepyceros melampus*): An immunocytochemical and computer image analysis study

P. VAN DER MERWE[1], D.G.A. MELTZER[1] and G. VAN ASWEGEN[2]

ABSTRACT

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Acute stress in the course of wildlife management has been intensively investigated. Chronic stress, on the contrary, has not been researched at all, probably due to the difficulty in measuring it as a result of the overriding effect of the physiological response to the restraining of wild animals. It was therefore decided to evaluate the use of immunocytochemistry, combined with computer image analysis to try and determine the magnitude of the structural changes of various hormone-secreting cells of the hypophysis. Since it was a pilot study to determine whether the combination of immunocytochemistry with computer image analysis could be of value to distinguish between two normally diverse groups, it was decided to compare the relative activity of prolactin secreting cells of lactating and non-lactating impala ewes. After transforming the prolactin immunoreactive area data by log10 to fall inside the parameters for kurtosis and skewness, a significant difference ($P < 0,05$, 5-% level, 2-tail) with the parametric *t*-test could be shown between the mean prolactin immunoreactive area of lactating (3,0751 μm^2) and non-lactating (3,0467 μm^2) ewes. However, the Pearson product moment ($r = 0,03$) showed that this difference may not be important for all practical reasons. This may be due to either sampling errors or limitations of computer image analysis, as it was often difficult to distinguish individual prolactin immunoreactive areas. Furthermore, a significant increase in the total prolactin immunoreactive areas of lactating ewes was also established. This technique, however, could distinguish between the hypophyses of lactating and non-lactating impala ewes, and with further refinement could be a useful tool in determining chronic stress in wildlife populations.

Keywords: *Aepyceros melampus*, chronic stress, computer image analysis, hypophysis, immunocytochemistry, impala, prolactin

INTRODUCTION

The precise estimation of blood hormone levels in wild animals is 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 (Ganhao, Hattingh, Pitts, Raath, De Klerk & De Vos 1988; Knox, Zeller & Hattingh 1993). Plasma hormone concentrations, for example cortisol, change rapidly as part of this physiological response, making the measurement of these concentrations difficult to interpret (Ganhao *et al.* 1988).

Where a pattern of disturbance and resultant stressors have been prolonged over months and years, chronic physiological changes may take place (Rampacek, Kraeling, Fonda & Barb 1984; Bruno, Olchovsky, White, Leidy, Song & Berelowitz 1990; Klemcke 1994; Parrott, Misson & De la Riva 1994). It has been postulated that these changes may lead to a disharmony within the hypothalamic-pituitary-axis characterized by some as a discorrelation in endocrine control (Przekop, Wolinska-Witort, Mateusiak, Sadowski

[1] Price Forbes Chair in Wildlife, Faculty of Veterinary Science, Private Bag X4, Onderstepoort, 0110 South Africa

[2] Department of Pharmacology, Potchefstroom University for Christian Higher Education, Potchefstroom, 2520 South Africa

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& Domanski 1984). It is suggested that disturbance of this central control of hormone secretion might affect the structure, storage function and activity of tropin hormone secreting cells in the hypophysis (Duvilanski, Zambruno, Seilicovich, Pisera, Lasaga, Del-C-Diaz, Belova, Rettori, McCann & Diaz-M-Del 1995).

Radioimmunoassay is generally employed to study the bioactive peptides of the hypophysis in plasma (Opel & Proudman 1984; Przekop *et al.* 1984; Rampacek *et al.* 1984; Worthy, Escreet, Renton, Eckersall, Douglas & Flint 1986; Colborn, Thompson Jr, Rahmanian & Roth 1991; Rojkittikhun, Uvnas-Moberg, Einarsson & Lundeheim 1991; Berardinelli, Godfrey, Adair, Lunstra, Byerley, Cardenas & Randel 1992; Van Nesselrooij, Kuper & Bosland 1992; Klemcke 1994; Parrott *et al.* 1994). Under veld conditions, radioimmunoassay is cumbersome as a specific controlled procedure for the sampling of plasma, and a wide variety of equipment and facilities, including cooling facilities, is needed (Gupta 1980; Day & Bolton 1982; Stupnicki 1982; Banky, Nagy & Halasz 1994).

Immunocytochemistry has been successfully used in visualising hormone secreting cells in the hypophysis of man (Stefaneanu, Kovaks, Lloyd, Scheithauer, Young, Sano & Jin 1992), rats (Van Nesselrooij *et al.* 1992), primates (Borson, Schatteman, Claude & Bothwell 1994), chickens (Lopez, Hargis, Dean & Porter 1995) and sheep (Pliska, Hari, Heiniger, Neuenschwander & Stranzinger 1992). This technique has an advantage in veld conditions as samples do not have to be collected under specific controlled conditions, and tissues collected can be immediately fixed in a preservative (Polak & Van Noorden 1987).

Computer assisted image analysis has been successfully employed to obtain prolactin and growth hormone immunoreactive sagittal area measurements in hypophyseal sections of turkeys. These measurements were expressed as a percentage of the total pituitary area (Ramesh, Proudman & Kuenzel 1995).

It was thus decided to evaluate the use of immunocytochemistry, combined with computer assisted image analysis, not only to visualize hypophysial 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 so as to validate it as a possible technique in estimating the presence of chronic stress in wildlife. As a pilot study to test this technique, it was decided to compare the relative activity of prolactin secreting cells of the hypophyses of lactating and non-lactating wild animals by statistically analyzing the difference in the total number and mean surface area of the stained immunoreactive images.

Impalas (*Aepyceros melampus*) are abundant in many nature reserves in South Africa and often population control measures are necessary to prevent overgrazing (Skinner & Smithers 1990). This creates a unique opportunity to study the physiology of this species, such as the effect of prolonged stressors, in order to apply it 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.

MATERIALS AND METHODS

Animals

Twenty adult impala ewes were culled in the southern part of the Kruger National Park. In order to eliminate physiological responses due to acute stress, all animals were culled by a neck shot, which severed the spinal cord (Van Nesselrooij *et al.* 1992). The ewes were divided into two groups, lactating ($n = 10$) and non-lactating ($n = 10$), by examining the udder for the presence of milk.

Tissue sampling

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 necropsy saw. 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. This exposed the floor of the cranial fossa, and the hypophysis could be dissected out using rat-tooth forceps and a scalpel.

Fixation and processing

Immediately following removal, the hypophyses were placed in Bouin's fluid at room temperature and left to fix for 12 h. They were then transferred to 30 % ethanol and sent to the laboratory where they were dehydrated in ethanol and embedded in paraffin wax (Polak & Van Noorden 1987). Twenty vertical sections (5 µm thick) were cut and floated onto slides pretreated with poly-L-lysine (Huang, Gibson, Facer, Gu & Polak 1983; Kotze & Van Aswegen 1990).

Immunocytochemistry

The paraffin sections were dewaxed and endogenous peroxidase blocked by treating the sections with 0,3% hydrogen peroxide in methanol for 30 min (Burns 1979). 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 for 10 min at room temperature with 10% pure swine serum (Burns 1979). The indirect peroxidase

and the peroxidase anti-peroxidase methods (Steward 1976; Sternberger 1986) were employed to demonstrate prolactin-containing cells. The primary antiserum, code 556, was supplied by Prof. J.M. Polak, Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, London, and used at a final dilution of 1:2000 to ensure optimal staining. Prolactin reaction sites were revealed by adding 3,3'-diaminobenzidine, which served as an electron donor to the peroxidase-anti-peroxidase complex, to the reaction. The subsequent redox reaction polymerized the 3,3'-diaminobenzidine to a brown insoluble substance, which is permanent and can be seen by light microscopy as described by Graham & Karnovsky (1966).

Controls for immunocytochemistry

The diluted primary antiserum was pre-absorbed by 20 µg/ml of its parent peptide, or alternatively, it was replaced by a non-immune serum.

Evaluation of prolactin immunoreactive areas

Ten randomly selected microscopic fields (150 000 µm^2) of ten sections of each hypophysis, where the staining produced adequate contrast to be detected by the image analysis system, were analized. All immunoreactive areas were counted and the surface area measured by means of computer assisted image analysis with a Cambridge Instruments Quantum 520 system. This system employs a video camera connected to a microscope to relay the image to a computer monitor for the analysis of the image. The prolactin immunoreactive areas, stained brown with the immunocytochemistry procedure, were then selected by the system, counted and analyzed. This gave the total prolactin immunoreactive areas counted in a field and the surface area (µm^2) of each prolactin immunoreactive area, which might comprise more than one cell, on a computer printout (Fig. 1).

RESULTS

The prolactin immunoreactive areas (µm^2) from the computer printout were statistically analyzed to determine the mean prolactin immunoreactive area for that field. Due to the skew distribution of the data, this was transformed by log10 to fall inside the acceptable limits for kurtosis and skewness. Further analysis of the two groups with the parametric *t*-test showed a significant difference between the mean immunoreactive area of lactating and non-lactating ewes ($P < 0,05$, 5-% level, 2-tail). However, the Pearson product moment ($r = 0,03$) showed that this difference might not be important for all practical reasons. A significant increase of the total prolactin immunoreactive areas for lactating ewes was also established. Results are summarized in Table 1.

DISCUSSION

Prolactin, a known lactogenic peptide hormone secreted by lactotrophs in the anterior hypophysis

TABLE 1 Total prolactin immunoreactive areas (PIA) counted, Mean prolactin immunoreactive surface area after transformation (mean PIA) and standard deviation

Group	Total PIA (µm^2)	Mean PIA	Standard deviation
Non-lactating ($n = 10$)	1090	3,0467	0,368
Lactating ($n = 10$)	1202	3,0751	0,368

FIG. 1 Individual prolactin immunoreactive areas in a non-lactating impala female. PAP x 1200

(Halmi 1983), is responsible for the development of the mammary glands as well as milk production (Meyer 1979; Worthy *et al.* 1986). These cells are active during lactation and become both hyperplastic and hypertrophic (Halmi 1983). 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 area recorded by computer assisted image analysis increased significantly in lactating impala ewes when compared to non-lactating ewes, rejecting the null-hypothesis of no difference in the prolactin immunoreactive areas of the two groups. The effect size, as calculated by the Pearson product moment, however, may render the results impractical. 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 error may have been compounded by the swiftness by which weaning occurs in impalas (Skinner & Smithers 1990) and the dramatic effect weaning has on the lowering of the level of circulating prolactin (Rojkittikhun *et al.* 1991). It may have been further exacerbated by external factors such as stress (Przekop *et al.* 1984; Sagrillo & Voogt 1991; Berardinelli *et al.* 1992; Martucci, Jessup, Gronert, Reitan & Clark 1992; Van Nesselrooij *et al.*1992; Banky *et al.* 1994; Parrott *et al.* 1994; Klemcke 1994), temperature (Colborn *et al.* 1991; Berardinelli *et al.* 1992; Narinder, Chaudhary & Singh 1992), exercise (Colborn *et al.* 1991) and particular limitations of computer image analysis, where it is often impossible to distinguish individual prolactin immunoreactive areas. Large confluent prolactin immunoreactive areas were often visualized which contributed to the skewness of the data (Fig. 2).

The significant increase in the total prolactin immunoreactive areas recorded can be explained either by hyperplasia or hypertrophy of lactotrophs, transdifferentiation of somatotrophs to lactotrophs or a combination of the above (Goluboff & Ezrin 1969; Stefaneanu *et al.* 1992; Ramesh *et al.* 1995).

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 mean prolactin immunoreactive area in the hypophysis. Further studies on more diverse groups, however, are needed in order to validate this technique.

Stress is of paramount importance in the management of wildlife populations and may result in significant losses due to mortalities (Knox *et al.* 1993). Acute stress in the course of wildlife management has been intensively investigated (Hofmeyer, Louw & Du Preez 1973; Franzmann, Flynn & Arneson 1975; Hofmeyer, Luchtenstein & Mostert 1976; Gericke, Hofmeyer & Louw 1978; Ganhao *et al.* 1988; Hattingh, Pitts & Ganhao 1989; Knox, Hattingh & Raath 1990; Knox, Hattingh & Raath 1991; Knox, Hattingh & Raath 1992; Williams, Rebar, Teclaw & Yoos 1995). Chronic stress, on the contrary, has only been researched in domesticated animals (Przekop *et al.* 1984; Rampacek *et al.* 1984; Klemcke 1994) as no references pertaining to wildlife could be found. This is probably due to the difficulty in measuring chronic stress in wildlife due to the overriding effect of the physiological response to restraining the animals to

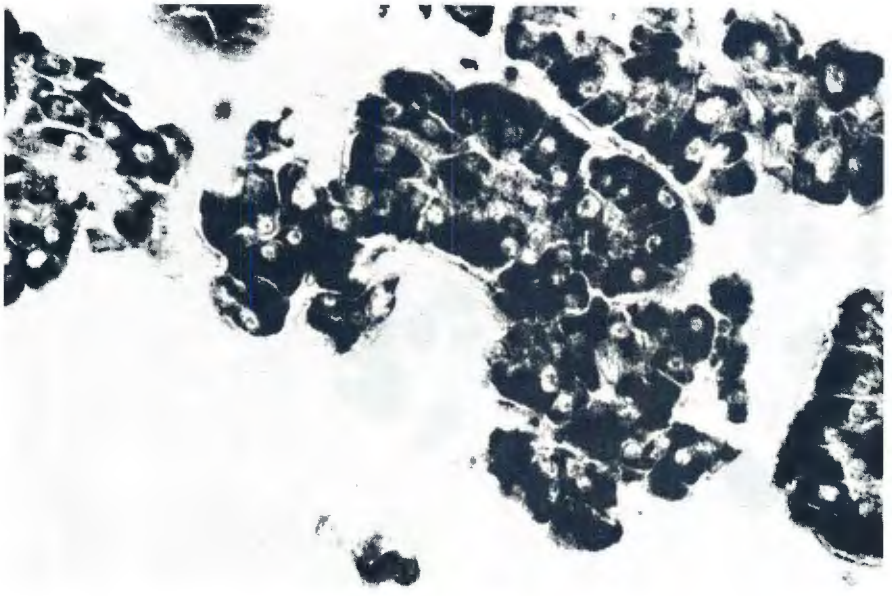

FIG. 2 Large confluent immunoreactive areas in a lactating impala female. PAP x 1200

be examined. Although many factors influence prolactin secretion, one of them is stress (Przekop *et al.* 1984; Sagrillo & Voogt 1991; Berardinelli *et al.* 1992; Martucci *et al.* 1992; Van Nesselrooij *et al.* 1992; Banky *et al.* 1994; Klemcke 1994; Parrott *et al.* 1994).

The methods employed in this investigation made it possible to estimate prolactin immunoreactive areas. However, the sampling methods need to be refined in order to obtain more accurate results. Further studies will take place to refine the sampling and computer assisted image analysis in an attempt to estimate the level of chronic stress in wildlife.

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