A multivariate approach to socio-ecological development and endocrine variance in the spotted hyaena, *Crocuta crocuta* Erxleben

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Abstract. This investigation of the socio-ecological development and endocrine variance in the spotted hyaena, *Crocuta crocuta,* illustrates that the complexity of a multivariate endocrine system cannot fully be understood using the concept of a class mean (μ). Any comprehensive investigation of a multivariate endocrine system should also include an analysis of variance (σ), as it may provide additional insights into the dynamics of an endocrine hypervolume. Mean cortisol concentrations could not differentiate between various social and reproductive categories (MANOVA); however, in males an analysis of variance (principal component analysis) indicated that the contrast between cortisol and androstenedione was the principle axis of variance once the androgen secreting ability had been accounted for. On the other hand, the same contrast was found to be the principal axis of variance in the female sub-sample, suggesting that cortisol may play a significant role in regulating the endocrine dynamics of this species, despite showing little variance in mean values among various social and reproductive categories.

Key words. Crocuta; multivariate; endocrine; MANOVA; principal component analysis.

"Natural science does not simply describe and explain nature; it is a part of the interplay between nature and ourselves; it describes nature as exposed to our method of questioning" .... (Heisenberg[1])

Most modern endocrinology textbooks agree that the maintenance of homeostasis or an integrated hormonal response in multicellular organisms is facilitated by the simultaneous monitoring of various endocrine components. The result is a plethora of synergistic and antagonistic endocrine actions that enable coordinated responses, metabolic fine tuning, and the attenuation or termination of such responses[2]. This multidimensional character of homeostatic endocrine systems has received little attention, and the popular trend is to investigate these phenomena using a reductionist approach. However, this latter approach has limitations when applied uncritically to biological specimens. The heterogenous nature of biological material means that biological complexity cannot fully be understood using the concept of a class mean (μ)[3,4]. This is due to the fact that the observed variance (σ) in biology is a reality, and should not simply be ascribed to differential boundary conditions or measurement errors, an assumption which is central to some branches of the physical and chemical sciences. In essence, biology should therefore be a science directed towards the description of the nature of biological variety or heterogeneity[3,4].

Neither the principles of multiple integration nor that of biological variance consistently form part of the modern endocrine discipline, which makes it difficult to formulate any holistic view of endocrine dynamics. This investigation into the endocrine correlates of social development in the spotted hyaena, *Crocuta crocuta,* is an attempt to incorporate these principles, and to achieve some understanding of the multivariate nature of endocrine systems. In order to investigate both trends in mean values as well as the nature of variance within a multidimensional hypervolume, the combined use of a multiple analysis of variance (MANOVA) and a principal component analysis (PCA) is recommended. MANOVA compares means among numerous treatments when a number of variables have been measured from each sample[22], whereas PCA defines the axes of greatest variance within any particular multidimensional data set[24].

The spotted hyaena social unit or clan comprises philopatric females (females stay and breed in their natal clans and do not disperse), their cubs, and three classes of males: resident natal males (RNM) born in the clan, peripheral immigrant males (PIM) that are in the process of dispersing, and central immigrant males (CIM) that have been accepted into a target clan and have procured mating rites[5]. The clan is organised in a near-linear social hierarchy with mature adult females being dominant, followed by other natal members, then CIMs and PIMs in that order[5,6]. This strict social hierarchy is established and maintained through agonistic interactions and plays an important role in establishing feeding priority as well as carcass division[5,7,8].

Plasma androgen concentrations in male spotted hyaenas are related to the dispersal stage of animals, with only those males that have successfully dispersed and have been accepted into target clans as mating males, having higher concentrations than females. On the other hand, neonatal females are exposed to higher titres of androstenedione which may play a role in the establishment of female dominance[9,10]. Although the high levels of intraspecific aggression in spotted hyaena society and the class related rank system suggests that social stress may play a significant role in regulating the endocrine dynamics of individual hyaenas, recent work demonstrated that the pattern of cortisol and prolactin secretion in males failed to support the 'social turmoil' hypothesis[11] as a general mechanism[12-14]. Animals experiencing social turmoil failed to show significant increases in mean cortisol or prolactin concentrations [12,14]. However, both these variables displayed large variances between individuals within the same reproductive and/or social categories, suggesting that another character, such as different styles of dominance or personality differences could be involved [15]. Moreover, lactating females had lower serum prolactin concentrations than all other reproductive categories, and it has been suggested that this enables prolonged lactation without interfering with ovarian activity [16-18].

Materials and methods

Animals. 51 spotted hyaenas with established social histories [10], and from various localities [10] in southern Africa were immobilized with Zoletil [19]. Subsequently animals were grouped into the following social categories: female cubs (FC, ≤ 1 year; $n = 6$), resident females (RF, > 1 year; $n = 16$), male cubs (MC, ≤ 1 year; $n = 14$), resident natal males (RNM; $n = 4$), peripheral immigrant males (PIM; $n = 5$) or central immigrant males (CIM; $n = 3$), reflecting the various dispersal stages of males from their natal clans [5].

Animals were sexed by scrotal palpation [20], and reproductive status was determined through external examination following Matthews [21]. Animals were grouped as belonging to one of the following reproductive categories: nulliparous female (NF; $n = 11$), parous female (PF; $n = 5$), lactating female (LF; $n = 6$), immature male (IM, ≤ 2 years; $n = 16$) and mature male (MM, > 2 years; $n = 10$). The single urogenital orifice in female spotted hyaenas as well as the distinct nipple development that occurs after the first litter make the distinction between nulliparous and parous females of this species a simple task [21].

Blood sampling and hormone analyses. Animals were subjected to once-off sampling following immobilization. Plasma and serum were collected from the cephalic vein using multi-sample needles and venoject evacuated tubes. Samples were stored upright at 4 °C until centrifuged for 10 min at 3000 rpm and stored at -20 °C until assayed. Plasma testosterone, androstenedione [25], oestradiol-17β [16], cortisol [13] and serum prolactin [14] were assayed using established procedures. Assay protocols and validations have been published elsewhere [13,14,16,25].

Intra-assay coefficients of variation for testosterone, androstenedione, oestradiol-17β, cortisol and prolactin were 7.8%; 7.3%; 12.5%; 6.7% and 2.0%, whereas inter-assay coefficients of variation for the same assays were 9.8%; 5.3%; 2.5%; 10.2% and 14.1% respectively.

Statistical methods. All statistical procedures used were in accordance with the principles described by Sokal and Rohlf [22]. Statistical manipulations were carried out on the University based mainframe computer using SAS Institute Inc. (Illinois, USA) software. The frequency distributions of all data sets were tested for normality, and variance for homoscedasticity. Where raw data failed to comply with the required criteria for parametric analyses, data transformations were employed in order to obtain normality and/or homoscedasticity. Multiple analysis of variance (MANOVA) was used to establish a comprehensive picture of the interactions between the means of the measured hormonal variables. Mean hormone concentrations among the various reproductive and social categories were compared to one another, and were used to test for the presence of a significant combined effect of the reproductive and social categories. The MANOVA was run using the SAS GLM procedure. Principle component analysis (PCA) on all the animals was used to establish the principle axes of variance for the hormonal hypervolume, and to identify any outliers in the data set. In addition, PCA effectively reduces dimensionality and therefore simplifies the interpretation and explanation of underlying patterns [24]. PCA was run using the SAS PRINCOMP procedure, first on the complete data set and then on males and females separately.

Results and discussion

MANOVA. Multiple analysis of variance (MANOVA) carries out the equivalent of an ANOVA in cases where a number of variables have been measured for several samples [22]. The mean concentrations of some hormones differed significanlty between the various reproductive categories ($F_{[15,97]} = 7.8$; $p < 0.001$) and between the social categories ($F_{[20,117]} = 2.6$; $p < 0.001$). Differences between Duncan groupings (those means that do not differ significantly from one other) are depicted in figure 1. A number of prominent features emerge from the analysis. A comparison of mean cortisol values failed to distinguish between reproductive and social categories, whereas prolactin had a limited resolution among the reproductive and social categories. Differences in mean androgen concentrations among the various categories reflect trends published earlier, with the CIMs being prominent among the social categories in terms of having significantly higher testosterone [10] concentrations than all the other categories (fig. 1 b). Likewise, both PFs and LFs among the reproductive categories (fig. 1 a), differed significantly from all other categories, for the circulating levels of oestradiol-17β [16] and prolactin [14] respectively, identifying them as other extreme endocrine conditions. Moreover, the categories showed no combined effect on the mean hormonal parameter estimates. This means that their effects could be evaluated separately.

Although a number of suspected developmental trends can be singled out, such as the testosterone concentrations that increase from the MCs, through the intermediate dispersal stages (RNMs and PIMs) to those animals that are responsible for matings in target clans following dispersal (CIMs), and the oestradiol-17β secreting ability of parous females, it still remains difficult to formulate any comprehensive view of the multi-dimensional char-

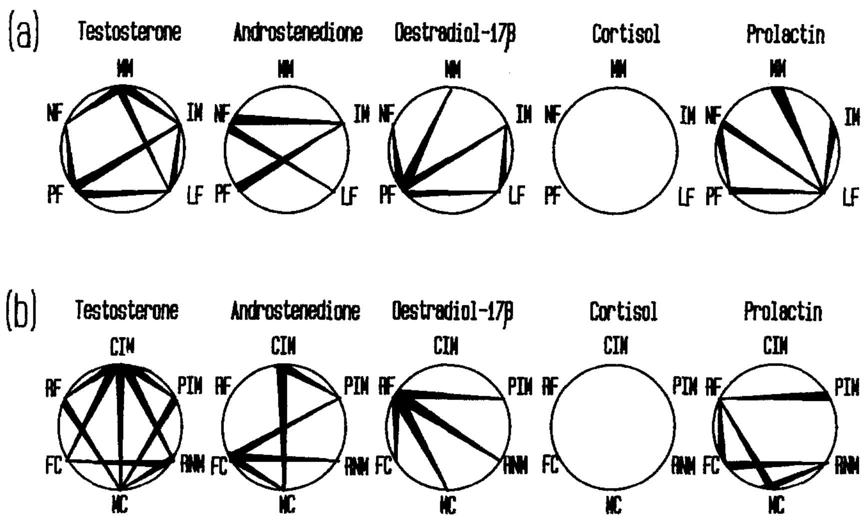

Figure 1. Results of the MANOVA which tested for significant differences in mean values ($\log_{10}$) among (a) reproductive categories and (b) social categories. The compared variables are depicted on the periphery of the circle, while connected variables indicate significant differences ($p < 0.05$) between categories. Thicker ends of the connecting elements point towards those categories that were found to have significantly higher mean values. MM = mature male, IM = immature male, LF = lactating female, PF = parous female, NF = nulliparous female, CIM = central immigrant male, PIM = peripheral immigrant male, RNM = resident natal male, MC = male cub, FC = female cub, RF = resident female.

acter of the endocrine dynamics of this species. In short, this technique represents a multi-dimensional confirmation of previously published results concerning endocrine variance in this species[10,13,14,16,25]. However, its significance lies in the fact that it compares means using a number of variables, and is therefore a useful way of analysing one component of biological complexity, namely that of class means (μ).

As stated earlier, using more than one classification system such as the reproductive and social categories used in the present study to compare trends in mean differences can be dangerous, as a combined effect of both classification systems may in operation. A multivariate analysis using both classification systems to test for mean differences would therefore represent a repetitive analysis of the same effect. Were a combined effect present, the classification system would have to be re-evaluated or re-described. As no combined effect or interaction between the two classification systems could be demonstrated in the present study, this danger is not an issue here. Furthermore, the MANOVA provides an easy method for identifying important trends in the mean values of any multivariate problem. It is clear from the graphical representation of the MANOVA (fig. 1) that the testosterone concentrations in CIMs, the oestradiol-17β concentrations in PFs and the prolactin levels in LFs represent extreme mean endocrine conditions in this species, whereas mean cortisol concentrations do not differ significantly between the various reproductive or social categories investigated.

Principle component analysis (PCA). In order to address the second component of biological complexity, namely variance (σ), PCA was used to gain some insight into the nature of multidimensional endocrine variance during the socio-ecological development of spotted hyaenas. PCA allows the definition of those axes responsible for the most variance within any n-dimensional hypervolume or data set. Both the nature of the principal axes of variance as well as the relative contribution of each axis to the total variance can be derived[24]. The first principal component (PC-1) of the complete sample (that axis which describes the most variance) consists of a strong total androgen effect, with both testosterone and androstenedione making large contributions to the total variance along this axis (see eigenvectors, table). The second PC contrasts high testosterone and cortisol concentrations against androstenedione. This in effect means that after the total androgen effect had been accounted for (PC-1) the main source of variation is between individuals with high testosterone and cortisol concentrations when compared with androstenedione concentrations. The third PC is a strong contrast between cortisol and testosterone, suggesting that when the variation from the first two PCs had been accounted for the biggest additional source of variance is between those individuals that have high cortisol concentrations compared with testosterone. A graphic representation of the variance around the first two PCs of the complete sample is given in figure 2. Although there is a lot of overlap between the sexes it does appear that the two sexes show variances in different directions. The males show a larger lateral distribution (PC-1) whereas the females show a large vertical distribution (PC-2). This suggests that the sexes should possibly be analysed separately.

The separate analysis of the sexes confirmed the trend observed in the above analysis, that the sexes were subject to different sources of variation, as their principle

Eigenvectors and eigenvalues for three PCAs performed on the complete hormonal hypervolume ($\log_{10}$) and the contributions of each to the total variation. Analyses were done on the total sample as well as for females and males separately.

Variables	Total sample			Males			Females		
ng/ml × $\log_{10}$	PC-1,	PC-2,	PC-3	PC-1,	PC-2,	PC-3	PC-1,	PC-2,	PC-3
Eigenvectors									
Testosterone	0.61	0.62	−0.46	0.70	−0.71	−0.06	−0.16	0.74	0.08
Androstenedione	0.73	−0.65	0.14	0.63	0.66	−0.38	0.77	0.16	0.47
Oestradiol-17β	0.23	0.05	0.01	0.11	0.08	0.03	−0.10	0.61	0.08
Cortisol	0.20	0.42	0.87	0.31	0.22	0.90	−0.55	−0.17	0.80
Prolactin	−0.02	−0.13	−0.12	−0.02	−0.03	−0.19	−0.26	−0.14	0.36
Eigenvalues	0.63	0.34	0.22	0.89	0.28	0.20	0.46	0.34	0.17
Contribution to total variation (%)	46	25	17	62	19	14	43	32	16
Cumulative (%)	46	71	88	62	81	95	43	75	91

axes differed significantly (table). In the females the PC-1 consisted of a contrast between androstenedione and cortisol concentrations. The PC-2 showed a strong testosterone and oestradiol-17β effect, whereas PC-3 had a large cortisol variance, and some contribution from androstenedione and prolactin. In males, PC-1 showed a strong testosterone and androstenedione effect, PC-2 was the result of a strong contrast between testosterone and androstenedione, while PC-3 was a strong contrast between cortisol and androstenedione concentrations.

The visual display of the first two PCs also revealed some interesting trends. In the males a clear developmental axis emerged with most of the male cubs (MC) concentrated at the top of the spread, and the mating males (CIMs) near the bottom right (fig. 3a). In between these two extremes there was a clear concentration of the RNMs with a tendency to cluster near the mating males. However, the PIMs showed little variance along PC-1, but showed a larger variance along PC-2 (fig. 3a) which suggests that their ability to produce both testosterone and androstenedione is affected.

The PC-1 calculated for males indicates that the total androgen concentration is the major hormonal variable describing the endocrine dynamics of male spotted hyaenas. The importance of this variable is confirmed by PC-2, which is an indication of the maturation of male testosterone secreting ability in preference to the precursor androstenedione, and probably reflects the switch in the testosterone: androstenedione ratio at puberty[10]. However, it also suggests that those males that are subjected to social stress on a regular basis (PIMs), show some loss in their ability to produce both testosterone and androstenedione when compared with the RNMs. When the androgen secreting ability of the male has been accounted for (PC-1 and PC-2), the major source of variation within the male sub-sample is a contrast between high cortisol and low androstenedione concentrations. Whether this variable indicates a change in the steroid secreting ability of the adrenal remains to be established, but the observed variance in the PIMs was the highest of all the adult males when PC-1 was plotted against PC-3,

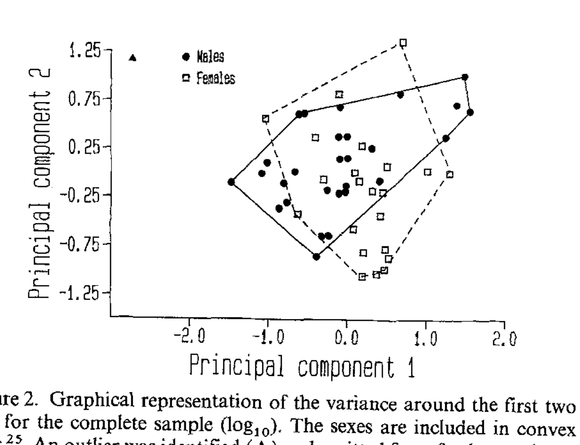

Figure 2. Graphical representation of the variance around the first two PCs for the complete sample ($\log_{10}$). The sexes are included in convex hulls[25]. An outlier was identified (▲) and omitted from further analyses.

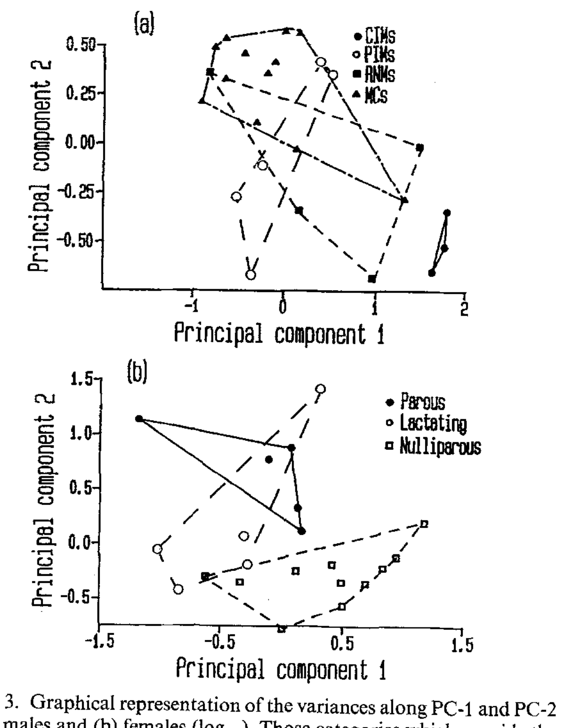

Figure 3. Graphical representation of the variances along PC-1 and PC-2 for (a) males and (b) females ($\log_{10}$). Those categories which provide the highest resolution in each sex are included in convex hulls[25]. See legend fig. 1 for abbreviations.

which suggests that there was a strong contrast between high cortisol and low androstenedione in this social category. Again, this may be indicative of some form of social inhibition of androgen secretion[10]. The MCs and the RNMs displayed a large variance in both PC-1 and PC-2, while the CIMs failed to show any significant variation along any of these axes. This could be an effect of sample size although there does seem to be a clear clustering of this latter category, suggesting an extreme endocrine condition which is associated with little variance. Females, on the other hand, showed a clear separation between the NFs and the adult females. Both the NFs and the PFs showed large variances along PC-1, whereas the LFs showed their largest variance along PC-2. The PC-1 for the female sub-sample which contrasts high androstenedione with low cortisol levels is similar to the PC-3 defined for males, however, the largest variance along this component is shown among the FCs and PFs. This suggests that there is an inverse relationship in terms of the ability to secrete these hormones, and again as was found for males, it may be that this is an indication of a change in steroid secreting ability, possibly in the adrenal. The LFs showed their largest variance along PC-2 which has a strong testosterone and oestradiol-17β effect and a slight contrast with the stress-related hormones cortisol and prolactin[15, 23]. This later observation is probably a function of the low prolactin levels found in this category (see MANOVA), but it also shows that a significant degree of ovarian activity occurs in this reproductive category. Further confirmation for the presence of significant ovarian activity during lactation in this species[16] is provided by the significant variance in the amount of testosterone and oestradiol-17β produced in this category. At this stage it is difficult to interpret PC-3 in the females which comprises a strong cortisol effect and some positive contributions from the hormones androstenedione and prolactin, but it could also be a stress-related variable.

In conclusion, the MANOVA performed in the present study identified mean testosterone concentrations in the CIMs, mean oestradiol-17β levels in PFs, and mean prolactin levels in LFs as extreme endocrine conditions for this species. However, in an investigation of multidimensional variance, the ability to secrete large quantities of androgens was identified as the most significant variant describing male social development. Furthermore, the nature of androgen variance suggests that the ability to secrete androgens is strongly related to circulating cortisol concentrations. The endocrine dynamics of female social development appears to be quite different. Although mean cortisol levels were not able to distinguish between any of the reproductive or social categories, the most significant axis of endocrine variance found in females was a strong contrast between androstenedione and cortisol concentrations, suggesting that the stress-related hormone may play an important role in regulating the dynamics of endocrine interaction. Although an adrenal origin for this pattern seems logical, this aspect still needs to be investigated. The patterns identified in this multivariate investigation of endocrine variation during the social development of the spotted hyaena, clearly illustrates that the comparison of means (MANOVA) and an investigation of the nature of hormonal variation (PCA) identify different aspects of the total endocrine dynamics of the species. Therefore, in order to achieve any comprehensive understanding of the dynamics of an endocrine hypervolume, an analysis of trends in mean hormonal values (μ) as well as the nature of multidimensional endocrine variance (σ) should be investigated.

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