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FAILURE OF GLUCOCORTICOID FEEDBACK DURING BREEDING IN THE MALE RED-TAILED PHASCOGALE *PHASCOGALE CALURA* (MARSUPIALIA: DASYURIDAE)

ADRIAN J. BRADLEY
Department of Zoology, University of Tasmania, Box 252C, GPO Hobart, Tasmania 7000, Australia

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Summary—An investigation was made into the factors which lead to an elevation in plasma free cortisol concentration during the last weeks of life of males in natural populations of the red-tailed phascogale *Phascogale calura*. The dexamethasone suppression-test was employed to examine the glucocorticoid feedback control of plasma cortisol both before and during the breeding season.

In both sexes ACTH alone or in combination with dexamethasone caused an elevation in the plasma concentration of cortisol, corticosterone and free cortisol. Dexamethasone administration in both males and females resulted in significant decreases in the plasma concentration of each of the glucocorticoid groups both before and during the first week of the breeding season (June and early July), however during the last week of breeding (late July) dexamethasone decreased the plasma glucocorticoid concentration of females but not of males. Administration of ACTH caused a significant elevation in the plasma cortisol concentration in all groups. However, the magnitude of this response diminished with time in both sexes.

Dexamethasone treatment resulted in a decrease in the plasma testosterone concentration in males before and early in the breeding season however toward the end of breeding this effect was abolished.

It is apparent that towards the end of the breeding season and during the last week of life of the males, glucocorticoid feedback control of ACTH is almost abolished. These changes, which occur only in the males late in the breeding season and near the time of their disappearance from the population, are consistent with a condition known as end organ resistance to steroid hormones.

INTRODUCTION

In recent years considerable interest has been focussed on several species of small Australian marsupials belonging to the family of marsupial carnivores, the Dasyuridae, where a short winter mating period is terminated by a synchronous and total male mortality. Studies of *Antechinus stuartii* [1, 2], *Antechinus swainsonii* and *Antechinus flavipes* [3, 4] and *Phascogale calura* [5,6] have established that a major factor in the male decline is an androgen-dependent decrease in corticosteroid binding globulin (CBG) concentration just prior to and during the breeding season. The breeding season in *P. calura* occurs during july each year and lasts for approximately three weeks after which all of the males disappear from the population and presumably die. These changes which occur in males but not in females coincide with an elevation in the plasma glucocorticoid concentration, the net result being a large increase in the concentration of plasma free glucocorticoid.

The demise of the males seems to be related to suppression of both immune and inflammatory reactions as a result of exposure of tissues to high concentrations of free glucocorticoid. This then results in an increased incidence of endoparasite and pathogenic infections and more consistently ulceration and haemorrhage from the gastric mucosa [2, 3, 5, 7]. The morphological and functional changes in males appear to conform to the "diseases of adaptation" of Selye [8] while the seemingly precarious life-history pattern may be considered to be an exaggerated example of a reproductive strategy which is primarily concerned with reproductive success but with deleterious effects resulting from the accompanying endocrine changes.

The dexamethasone suppression-test [9] has been used to show that the marked increase which occurs in the concentration of plasma free glucocorticoid during the last weeks of life of male *Antechinus swainsonii* resulted from a failure of the glucocorticoid feedback mechanism [10].

Since free glucocorticoids in the plasma are considered to be biologically active [11, 12] the large increases in both plasma free cortisol and corticosterone in *P. calura* males during the last two weeks before they disappear from the population [5] suggests that the negative feedback control of ACTH is affected.

This investigation was carried out to see whether the terminal elevation in the plasma free cortisol concentration in male *P. calura* could be explained by a failure of glucocorticoid feedback.

EXPERIMENTAL

Isotopes and chemicals

[1,2,6,7-^{3}H]Cortisol (85 Ci mmol^{-1}), [1,2,6,7-^{3}H]corticosterone (94 Ci mmol^{-1}) and [1,2,6,7-^{3}H]testosterone (88 Ci mmol^{-1}), were purchased from the Radiochemical Centre, Amersham, England, [1,2-^{3}H]11-deoxycortisol (38.5 Ci mmol^{-1}) was purchased from New England Nuclear, Boston, Mass. and [4-^{14}C]-testosterone (49 mCi mmol^{-1}) was purchased from Australian Radioisotopes, Sydney, Australia). Non-radioactive steroids were obtained from Sigma Chemical Co. (St Louis, Mo.) All other reagents used were of analytical grade and purchased from commercial sources. Dexamethasone (Decadron Phosphate 0.2 mg kg^{-1}) was supplied by Merck, Sharpe and Dohme, Australia and ACTH (ACTH purified repository corticotropin 40 IU ml^{-1}), from Arnolds of Reading Pty Ltd, Victoria, Australia.

Animals

Animals were captured in Elliott folding aluminium live traps (33 × 10 × 10 cm; Elliott Scientific Equipment, Upwey, Victoria) in Yornaning Nature Reserve located about 12 km WNW of Wickepin in the SW Wheatbelt of Western Australia. Traps were baited with a mixture of peanut paste and rolled oats and were prepared in such a way that hypothermia could be avoided in animals trapped during winter. Plastic bags were placed over the traps and shredded paper was added to provide nesting material.

Blood sampling

Small blood samples (approx. 0.1–0.2 ml) were collected by infra-orbital sinus puncture [13] within 1 min of disturbing the animal in the trap. There was no evidence either of infection or residual damage and the same animals could be captured repeatedly on subsequent occasions. Blood was taken through a heparinised micro-haematocrit tube (Clay Adams, Parsippany, N.J.) and collected in a 1.5 ml polypropylene centrifuge tube which contained a small amount of lithium heparinate (Sigma). Within 30 min blood was centrifuged and the plasma was withdrawn and stored at −25°C prior to analysis.

Assessment of adrenal reserves

At intervals groups of animals were removed from the field and held for 24 h for measurement of adrenal reserves. The three periods when animals were removed from the field for testing were late June, early July and late July. During the experimental period animals were transported 20 km to a field station where they were held individually in Elliott traps which contained moistened puppy chow (Robert Harper and Co., Melbourne, Australia) and some shredded paper.

On the day of capture animals of each sex were divided into four groups of five animals. All injections of 0.10 ml were given i.m. into the thigh. Animals were injected between 19.30 and 20.00 h with either saline, dexamethasone (0.2 mg kg^{-1}), or ACTH (100 IU kg^{-1}) and again between 23.30 and 24.00 h with one of the above. Different groups thus received one of these combinations: saline–saline (controls) dexamethasone–saline, saline–ACTH or dexamethasone–ACTH. Small blood samples were taken 5 h after the second injection. The dose of dexamethasone used was within the range reported to cause suppression of stress-induced increases in the concentration of plasma-glucocorticoid in several species of mammal [14–17]. The ACTH dose chosen was based upon studies with another small dasyurid *Antechinus stuartii* where sensitivity to the glucocorticoid stimulating action of β-ACTH (1-24) was less than for eutherian mammals [18].

Production of steroid antisera

Antibodies to cortisol, corticosterone and testosterone were raised in New Zealand white rabbits. Steroid conjugates 25 mg (Sigma) consisted of hydrocortisone-3-O-carboxymethyloxime:BSA, testosterone-3-O-carboxymethyloxime:BSA and corticosterone-21-hemisuccinate:BSA with steroid:BSA ratios of 13, 29 and 24 respectively. Each conjugate was dissolved in 6 ml of 0.9% saline containing 0.01% thimerosal (Sigma). Nine rabbits, three per

group, were given monthly injections of an emulsion consisting of 300 μl steroid conjugate and 300 μl Freund's complete adjuvant (Commonwealth Serum Laboratories, Australia). Injections were given i.d. at multiple sites in the suprascapular region. Monthly booster injections were continued for three months, and 10 days after the last injection 45 ml blood was withdrawn from a marginal vein of the ear. Blood was allowed to clot overnight then the sera were separated and either lyophylised or stored in 1 ml lots at -25°C.

Assessment of antisera

A phosphate buffer (0.05 M, pH 7.4) containing 0.06% bovine albumin [bovine albumin fraction V, Commonwealth Serum Laboratories (CSL), Melbourne, Australia], 0.01% normal human immunoglobulin (CSL) and 0.01% thimerosal (Sigma) was used routinely. The separation of the antibody bound and free steroid was achieved by the addition of 0.5 ml dextran coated charcoal suspension (0.5%, w/v) made up in assay buffer but without the added protein, incubation for 10 min then centrifugation at 3000 rpm for 10 min at 4°C.

Serial dilutions of each antiserum were incubated with the appropriate labelled and unlabelled steroid for between 8 and 10 h at 4°C in a total volume of 0.1 ml prior to separation of free from bound steroid. The antibody working titre was defined as the dilution giving 50% binding of the tracer.

Standard curves were constructed by incubating the appropriate steroid in 2-fold concentration increases between 0 and 800 pg with 0.1 ml of the diluted antiserum for 8–10 h prior to separation and counting.

Cross-reactivities are expressed as a percentage and calculated [19]. The affinity constants (K_a) for each antiserum were estimated from the standard curve data using Scatchard plots [20].

Chromatography

Plasma samples of 40–50 μl were extracted and chromatographed for the determination of androgens [2] and glucocorticoids [5].

Plasma albumin concentration

Plasma albumin was isolated from a pool of *P. calura* plasma as solution V using method 10 of Cohn *et al.* [21] and purity was confirmed by polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue G (Sigma). The total protein concentration of the isolated albumin was established [22] while plasma albumin concentrations were determined routinely [23] after scaling down to accommodate plasma samples of 10 μl. Sample absorbance was determined using a Pye Unicam SP6-550 Spectrophotometer (Pye Unicam Ltd, Cambridge, England).

Steroid–protein interactions

The binding constants for the steroid–protein interactions in the plasma of *P. calura* and the methods employed in their determination have been described elsewhere [5]. Plasma-corticosteroid binding globulin (CBG) binding capacity was measured by the gel filtration method of [24] and calculations of the partitioning of cortisol into CBG bound, albumin bound and free compartments were made at 36°C in 1:5 diluted plasma by the micro-equilibrium dialysis method of Englund *et al.* [25] and using Scatchard analysis of the data [20] and the correction of Slaunwhite and Rosenthal [26]. For cortisol the high affinity binding constant (K_a) of 4.36×10^7 l/mol was used while for the cortisol–albumin binding the constant used was 0.39×10^4 l/mol. No high affinity binding has been detected in the plasma of *P. calura* for either testosterone, 5α-dihydrotostosterone, oestradiol or aldosterone.

Measurement of radioactivity

Radioactivity was counted using a Beckman LS 5801 liquid scintillation spectrometer with automatic quench correction and assay results were than computed automatically using an RIA program. Samples were counted in Ecoscint (National Diagnostics, Somerville, N.J.).

Statistics

Data are expressed as mean $\pm$ SEM and tested by analysis of variance after Bartlett's test for homogeneity of variance. Groups in which variance was not homogeneous were log transformed prior to analysis. Comparisons between individual groups were made using Scheffé's F-test [27]. The null hypothesis was rejected at $P < 0.05$.

RESULTS

Assessment of antisera

Details of one of each of the steroid antisera are given in Table 1, including working titre, sensitivity, inter- and intra-assay variation and cross reactivity with other steroids.

Table 1. Steroid cross-reactions (in %) with antisera used in radioimmunoassays

	Anti-F F319	Anti-B B23112	Anti-T T3279
Cortisol	100	16.2	0.9
Cortisone	1.5	25.5	1.1
Corticosterone	10.4	100	1.6
11-Deoxycortisol	1.7	7.9	1.2
11-Deoxycorticosterone	19.5	7.4	1.2
Progesterone	3.2	26.9	2.3
Aldosterone	2.1	0.9	2.2
Androstenedione	1.9	1.6	6.2
Dihydrotestosterone	2.6	7.9	28.6
Testosterone	2.8	7.6	100
Epitestosterone	0.9	1.3	1.9
Estradiol	0.1	0.2	1.5
Titer used	6000	8000	5000
Sensitivity (pmol)	0.03	0.04	0.03
Variability (c.v.%)			
intra-assay	6.8	7.0	6.9
inter-assay	6.9	7.1	7.0
K_a (l/mol)	4.2×10^9	5.1×10^9	3.7×10^9

Anti-F, anti-cortisol; anti-B, anti-corticosterone; anti-T, anti-testosterone.

Plasma glucocorticoids

Plasma cortisol, corticosterone and free cortisol concentrations of both males and females in response to the experimental treatments are shown in Fig. 1. During each of the three test periods dexamethasone was effective in causing a significant depression in plasma cortisol, corticosterone and free cortisol concentration in the females. This pattern was repeated in the males in the first two test periods but not during the late July test period when only a very slight decrease occurred in the concentration of each steroid. Treatment of both males and females with saline plus ACTH caused significant increases in the concentration of cortisol, corticosterone and free cortisol during each of the test periods however there were some differences in the magnitude of this rise both between sexes and between test periods.

In the groups given dexamethasone plus ACTH some decline in responsiveness was observed between the three test periods however in each case the values were significantly higher than in the controls for the corresponding period. In the males during the first two test periods the saline plus ACTH treatment resulted in significantly greater rises in the concentration of both hormones than did the dexamethasone plus ACTH treatment. In late July the plasma corticosterone concentration in both the saline plus ACTH and the dexamethasone plus ACTH groups were significantly higher in the females than in the males while the plasma free cortisol concentration in the males was significantly higher than that in the females.

Plasma CBG binding capacity

The plasma CBG binding values for females during the three periods June, early July and late July were 262 ± 11, 235 ± 13 and 211 ± 15 nM while corresponding values for the males were 156 ± 9, 96 ± 11 and 82 ± 9 nM respectively. These decreases in CBG binding capacity between June and late July were significant in both the females ($P < 0.05$) and and males ($P < 0.01$). Determinations were made on plasma from ten individuals of each sex during each period.

Plasma albumin

The plasma albumin concentrations for females during the three periods June, early July and late July were 3.9 ± 0.2, 3.9 ± 0.3 and 4.1 ± 0.3 g dl^{-1} while corresponding values for the males were 4.2 ± 0.3, 4.2 ± 0.2 and 4.0 ± 0.3 g dl^{-1} respectively. Determinations were made on plasma from ten individuals of each sex during each period.

Plasma androgens

The changes in the plasma testosterone concentration in the males in response to treatment are shown in Fig. 2. The dexamethasone plus saline treatment caused a significant decrease in the plasma testosterone concentration in both June and early July but this was reversed in late July where a slight but insignificant increase occurred. The dexamethasone plus ACTH treatment also caused a significant decrease in the plasma testosterone during early July. The plasma testosterone concentration in the control groups increased progressively with time so that the value during late July was significantly higher than the value during June ($P < 0.05$).

DISCUSSION

The mechanism which allows the maintenance of elevated plasma free cortisol concentrations in small dasyurid marsupial males near the time at which they disappear from the population has remained an enigma [1, 2, 3, 5, 28]. This phenomenon may, in part, be explained by a failure of glucocorticoid feedback in male *A. swainsonii* [10] however the mechanism by which this occurs has yet to be explained.

The dasyurid marsupial species which show the post-mating mortality of males all show clear evidence of glucocorticoid hormone excess involving disturbances of nitrogen

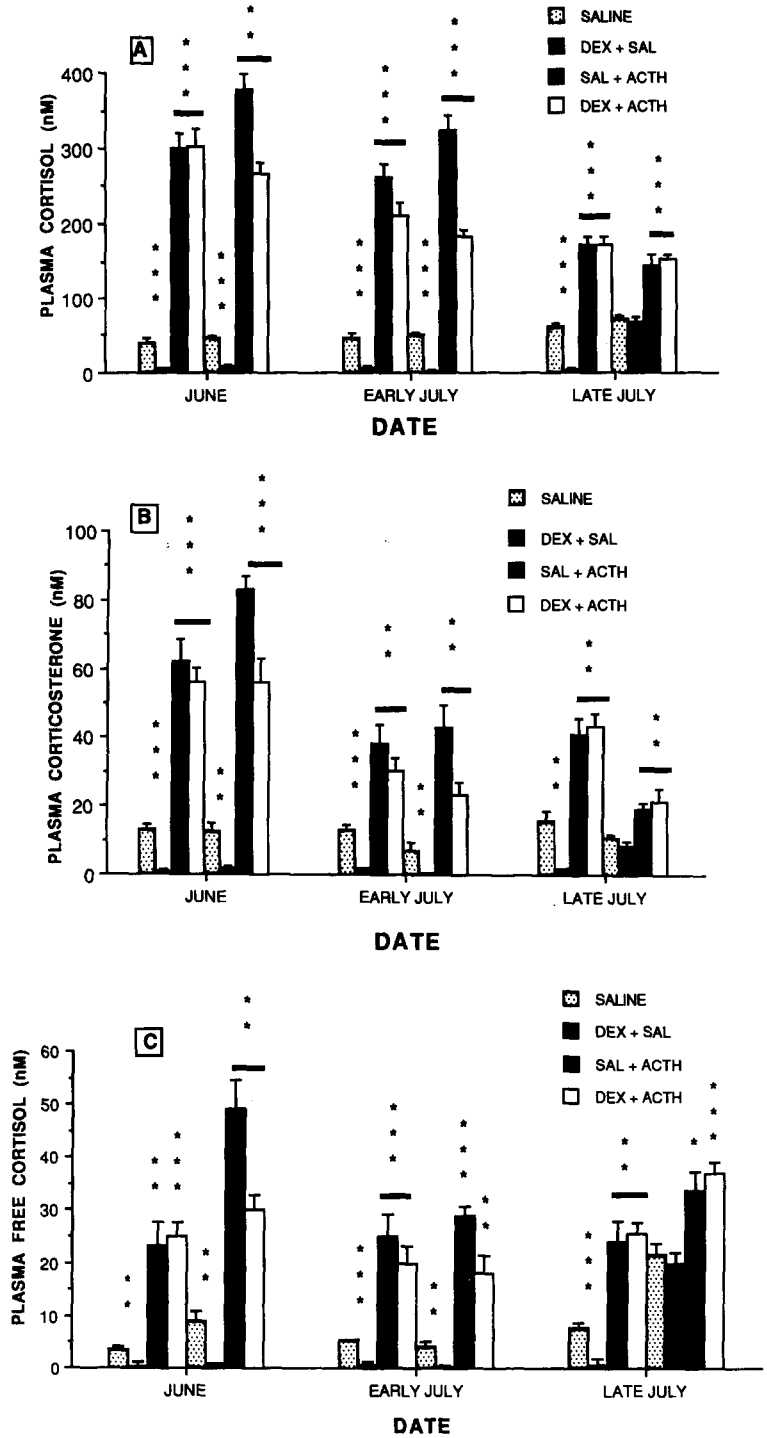

Fig. 1. Effect of saline (control), dexamethasone (DEX), ACTH and combined treatments on plasma concentrations of cortisol, corticosterone and free cortisol at different times before and during the breeding season in *P. calura*. Within each month the four histogram bars to the left are for females while those to the right are for males. Each point represents the group mean ± SEM with $n = 5$ animals. Significance compared with the respective controls is denoted thus $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

metabolism [29–31] and suppression of the immune system [2, 4–7, 32] and haemorrhage from gastric ulcers [2, 5, 7, 18]. The increases in plasma free cortisol and testosterone concentration in *P. calura* and *Antechinus* spp. are consistent with the presence of an intact hypothalamic–adrenal axis with functioning feedback mechanisms and the progressive development of resistance which affects the pars distalis and hypothalamus more than it does the

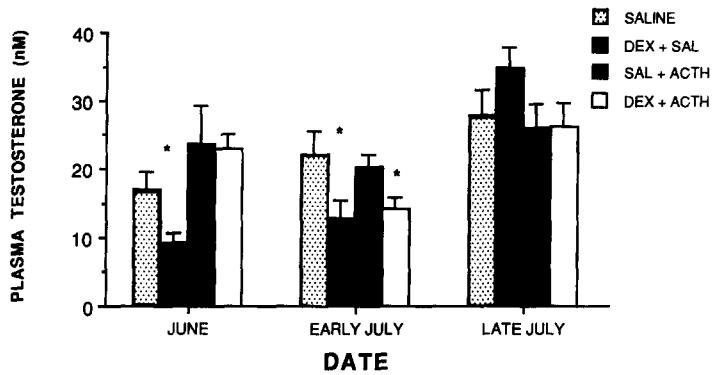

Fig. 2. Effect of saline (control), dexamethasone (DEX), ACTH and combined treatments on plasma concentration of testosterone at different times before and during the breeding season in *P. calura* males. Each point represents the group mean ± SEM with $n = 5$ animals. Significance compared with the respective controls is denoted thus $^*P < 0.05$.

other tissues. The possibility must also be considered that the elevation in plasma cortisol, and possibly testosterone, is the result of a diminished metabolic clearance rate (MCR) while the production rate (PR) may remain normal or even be slightly reduced. Findings similar to this have been made in studies on man [33], Pacific salmon [34] and Atlantic cod [35]. Until studies of MCR and PR are carried out in moribund dasyurid marsupial males, this explanation cannot be discounted. However a common finding of adrenocortical hypertrophy during the last weeks of life in the males of several of these species [2, 18 and Bradley (unpublished data)] coinciding with the elevation in plasma free cortisol concentration is consistent, at least, with an increase in PR.

Dexamethasone suppression-tests [9] carried out in June, early July and late July indicate that in the males during the first two periods the normal feedback mechanism was operative, however by late July the failure of dexamethasone to suppress glucocorticoid secretion suggests that a major change had occurred in the mechanism. This finding is similar to a previous study [10] where a failure of glucocorticoid feedback in male *A. swainsonii* occurred at the beginning of the mating period and within one month of their disappearance from the population. In *P. calura* however, the failure of feedback was detected seven days prior to the disappearance of the last of the males but the system behaved normally 20 days prior to this phenomenon. The precise cause of the failure in the glucocorticoid feedback mechanism toward the end of the mating period which presumably results in the exposure of tissues to high concentrations of plasma free glucocorticoids has yet to be established.

It is clear from this study on *P. calura* that the general changes in responsiveness of the pituitary–adrenal axis bear a striking resemblance to the changes described for *A. swainsonii.* The most notable feature was the almost total lack of effect of dexamethasone in lowering the plasma cortisol concentration in *P. calura* males in late July. This was in marked contrast to the effect of dexamethasone in females at this time and to the effect in both sexes at the earlier dates. Quite clearly a major change has occurred in the glucocorticoid feedback control. The other significant change concerned the response of both sexes to ACTH. In late July ACTH administration resulted in a significant elevation in the plasma free cortisol concentration in both sexes however the magnitude of this response was much less than was seen at the two earlier dates. This diminished response to ACTH is interesting and was not seen in the *A. swainsonii* study [10]. Diminished responsiveness to ACTH following prolonged ACTH treatment has been reported for *in vitro* studies using rabbit adrenal tissue [36]. Since a diminished response to ACTH is seen both in male and female *P. calura* this is consistent with prolonged stimulation of the pituitary–adrenal axis. Such stimulation would be expected to cause a greater increment in the plasma free cortisol concentration of males than it would in females because of the lower plasma concentration of corticosteroid binding globulin in the males [5].

In *P. calura* males the plasma testosterone concentration has been shown to rise significantly by mid June and to peak in late July just before the males disappear from the population [5] which also coincides with the failure of the glucocorticoid feedback. Failure of glucocorticoid feedback also occurred in *A.*

swainsonii when testosterone concentration was elevated [10] which leads to the speculation that the failure of glucocorticoid feedback might be related to the marked elevation in plasma testosterone concentration. There is some evidence from *in vivo* studies on the rat that adrenal progesterone secretion and its conversion to testosterone by steroidogenic enzymes in the cytoplasm of the Leydig cells may provide an alternative pathway for testosterone biosynthesis and may account for increased plasma testosterone levels during stress and mating [37] however it should be emphasised that this refers to the acute phase only. Terminal rises in plasma testosterone concentration, in fact, occur in males in all *Antechinus* spp. which share this unusual life-history pattern [2, 3, 38] and because of a lack of a sex hormone binding globulin (SHBG) much of this testosterone is presumed to be biologically active [39]. It has been suggested that these rises in plasma testosterone concentration in males may be associated with a disturbance to the steroidal feedback control of gonadotrophin secretion at the time of breeding [10]. If both the pituitary–adrenal and pituitary–gonadal axes are affected at this time, it is interesting to note that in *A. stuartii* a disturbance to spermatogenesis occurs up to three months prior to the peak in plasma testosterone concentration [40].

The decrease in plasma testosterone concentration following dexamethasone treatment in June and early July in *P. calura* is an effect which has been reported previously in the oppossum (*Didelphis marsupialis*) [41] and in *A. swainsonii* [10]. Dexamethasone administration is effective in suppressing testosterone in the olive baboon (*Paapio anubis*) [42] and a direct inhibitory effect of glucocorticoids upon testicular luteinising hormone receptor and steroidogenesis has been reported for the rat [43]. In cattle the effect may be due to a suppression of gonadotrophic hormone [44].

These seemingly uncontrolled rises in the concentration of plasma free cortisol and testosterone which occur in the wild in these small dasyurid marsupials bear striking resemblance to the condition known as end organ resistance to steroid hormones which has been described for cortisol [45–47], androgens [48], aldosterone [49] and progesterone [50]. Among the several New World primate species which show cortisol resistance the change is accompanied by a greatly increased plasma cortisol concentration, decreased cortisol binding globulin capacity and affinity, high levels of plasma and urinary free cortisol and marked resistance of ACTH suppression by dexamethasone but no physiological evidence of glucocorticoid hormone excess [45–47]. In these New World primate species end organ resistance is compensated for and manifests itself in the form of elevated plasma cortisol concentrations which might be expected since cortisol is essential for life in primates [45–46]. Since the adrenal glands appear to be essential for life in marsupials [51] rises in plasma cortisol concentration might also be expected to accompany end organ insensitivity.

It has been suggested [10] that this failure of glucocorticoid feedback in *A. swainsonii* may be related to the extremely intense level of sexual activity and aggressive interactions characteristic of the males of this species at the time of breeding. The changes which occur in moribund male *A. swainsonii* and *P. calura* resemble more the severe form of primary cortisol resistance in man [47] rather than partial or compensated end organ resistance [52]. Further studies on MCR, PR and steroid receptors are obviously necessary in moribund male *A. swainsonii* and *P. calura* to determine the reasons for the increase in the plasma concentration of cortisol and testosterone. A failure in the steroid feedback mechanism may, in fact, occur but this may be accompanied by a decrease in the sensitivity of the target tissues to the action of trophic hormones.

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