The Activity and Functioning of the Hypothalamic-Pituitary-Adrenal Axis of the Red Deer Stag (*Cervus elaphus*)

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This thesis is dedicated to my family and friends who's support and enthusiasm made all the difference.

Declaration

I declare that this thesis is a record of my own work.

John Ronald Ingram

Abstract

The activity and functioning of the HPA axis was investigated in red deer stags under basal and stress induced conditions. In order to overcome the confounding effects of stress inherent in standard methods of blood collection, a portable remote blood sampling device ("DracPac") was developed and tested. Stags were blood sampled remotely during restraint in a mechanical crush and subsequent recovery at pasture. Cortisol, haematocrit, glucose and lactate levels were elevated during restraint and declined significantly following release to pasture.

The DracPac device was used to deliver *i.v.* a range of $ACTH_{1-24}$ doses (1-64 IU/100kg) and collect blood for cortisol determination from red deer stags. ACTH significantly elevated plasma cortisol concentrations, with mean peak concentrations occurring 20 to 40 min post-infusion. Duration of the response (ranging from 80 to 160 min), was dose dependent, whereas cortisol concentration plateau with doses above 4 IU.

Seasonal changes in the activity and responsiveness of the adrenal gland in red deer stags were quantified by measuring 24 h endogenous cortisol secretory profiles and plasma cortisol responses to either administration of exogenous ACTH or a standardised stressor during November (period of velvet growth), February (pre-rut), April (mid-rut) and July (post-rut) (southern hemisphere) using a remote blood sampling device (DracPac). Ultradian, circadian and seasonal rhythms in the concentration of plasma cortisol were observed. Mean 24 h plasma cortisol concentrations were significantly higher in November than at other times of year. Peak cortisol concentrations following infusion of $ACTH_{1-24}$ (0.04 IU kg^{-1}) were also higher ($p < 0.05$) in November but lower ($p < 0.001$) in April than at other times of year.

The role of gonadal steroids in modulating the seasonal rhythm of HPA axis activity and function in the red deer stag was investigated. Basal secretion of cortisol and responses to administration of ACTH $_{1\text{-}24}$ (0.04 IU kg^{-1} live weight), CRH (25 ng kg^{-1} live weight) and dexamethasone (37 μg kg^{-1} live weight) were compared in castrated (n=6) and entire (n=6) male deer during the breeding season. A higher level of HPA axis basal activity and adrenal responsiveness to ACTH was seen in castrated compared with entire stags indicating that modulation of HPA axis function by testicular steroids occurs primarily at the level of the adrenal.

The effect of chronic social stress on behaviour and hypothalamic-pituitary-adrenal axis (HPA axis) activity and function was determined in 6 two-year-old red deer stags (Mixed) subjected to repeated mixing into unfamiliar herds. Following mixing basal cortisol concentrations declined, the proportion of unbound cortisol increased though free cortisol concentrations did not change. Responsiveness of the adrenal to ACTH declined, as did pituitary responsiveness to CRH.

HPA axis changes to stress occur at multiple levels within the axis. Acute stress is best assessed by frequent sampling of cortisol concentrations. Chronic stress can be best monitored by quantifying changes in HPA axis function (e.g. CRH challenge).

Acknowledgments

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Abbreviations

A	Adrenaline
ACH	Acetylcholine
ACTH	Adreno-corticotrophin
AL	Anterior Lobe
ANS	Automatic Nervous System
ANOVA	Analysis of Variance
AR	Androgen Receptors
AVP	Arginine Vasopressin
BNST	Bed Nucleus of the Stria Terminalis
BS	Brain Stem
CAMP	Cyclic Adenosine Mono-Phosphate
CBG	Corticosteroid-Binding Globulin
CLIP	Corticotropin-Like Intermediate Lobe Peptide
CNS	Central Nervous System
COMT	Catechol - o – methyltransferase
CRE	Cyclic AMP Response Element
CRH	Corticotrophin Releasing Hormone
CSCC	Cholesterol Side Chain Cleavage
DE	Debranching Enzyme
END	Endorphin
ENK	Enkephalin
FSH	Follicle Stimulating Hormone
g	gram
GABA	Gamma-aminobutyric Acid
GAS	General Adaptation Syndrome
GH	Growth Hormone
GnRH	Gonadotrophin Hormone Releasing Hormone
GR	Gluccorticoid
GRE	Glutocorticoid Response Element
HDL	High Density Lipoprotein

HPA	Hypothalmic-Pituitary-Adrenal
HGP	Hypothalmic-Pituitary-Gonadal
IL	Intermediate Lobe
JP	Joining Peptide
kg	kilogram
LC-NA	Locus Caeruleus-Noradrenaline
LDL	Low Density Lipoprotein
LH	Luteinising Hormone
MAO	Monoamine oxidase
mm	millimetre
MSH	α - Melanotropin
MPOA	Medial Pre-Optic Area
MR	Mineralcorticoid
mRNA	Messenger Ribonucleic Acid
n	number
NA	Noradrenaline
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
ng/ml	nanograms per millilitre
NL	Neutral Lobe
N-POC	N – Terminal Peptide
ns	not significant
OT	Oxytocin
P	probability value
PHI	Peptide Histidine-Isoleucine
PKA	Negative Logarithm of the Affinity Constant
POMC	Pro-Opiomelanocortin
PNS	Parasympathetic Nervous System
PRL	Prolactin
PVN	Paraventricular Nucleus
SAM	Sympathoadrenomedullary
SED	standard error of the difference
SEM	standard error of the mean

SON	Supraoptic Nucleus
StAR	Steriodogenic Acute Regulatory Protein
TNF	Tumour Necrosis Factor
TSH	Thyrotropin
VFI	Voluntary Food Intake
VIP	Vasoactive Intestinal Peptide
VMA	Vanillylmandelic Acid
VP	Vasopressin
yMSH	y – Melanocyte Stimulating Hormone
ß-END	ß-Endorphin
ß-LPH	ß-Lipotropin
3ß-HSD	3 – ß- Hydroxysteroid dehydrogenase
11-ß-HSD	11- ß – Hydroxysteroid dehydrogenase
µl	microlitre
µm	micrometre

List of Publications

The following is a list of publications that have arisen from work reported in this thesis.

Ingram J.R., Matthews L.R. and McDonald R.M. 1994. A stress free blood sampling technique for free ranging animals. *Proceedings of the New Zealand Society of Animal Production.* **54** 39-42. *(Basis for Chapter 3)*

Ingram J.R., Matthews L.R., Carragher J.F., and Schaare P.R. 1997. Plasma cortisol responses to remote adrenocorticotrophic hormone (ACTH) infusion in free ranging red deer (*Cervus elaphus*). *Domestic Animal Endocrinology.* **14** 63-71. *(Basis for Chapter 4)*

Ingram J.R., Crockford J.N., Matthews L.R. 1999. Ultradian, circadian and seasonal rhythms in cortisol secretion and adrenal responsiveness to ACTH and acute stress in unrestrained red deer stags (*Cervus elaphus*). *Journal of Endocrinology* **162** 289-300. *(Basis for Chapter 5)*

Cook CJ, Mellor DJ, Harris PJ, Ingram JR and Matthews LR. 2000. Hands on and hands off measurement of stress. In: Moberg GP and Mench JA (eds.) *The Biology of Animal Stress: Assessment and Implications for Welfare.* CAB International, Wallingford, Oxon, UK, pp 123-146.

CHAPTER 1 GENERAL INTRODUCTION

1.1 INTRODUCTION

The farming of red deer (*Cervus elaphus*) for venison and velvet antler production has progressed rapidly since the establishment of deer farming using captured feral animals in the 1970's. Deer management procedures have continued to evolve to better suit the requirements of deer using specific nutritional and reproductive strategies based on decades of scientific research. The reduction of stress is an important aspect of improved management, reducing animal welfare problems and increasing the efficiency of production systems. The hypothalamic-pituitary-adrenal (HPA axis) is a key component of the stress response. Changes in the activity and functioning of the HPA axis are routinely used to quantify stress in farm animals (Barnett & Hemsworth, 1990, Fraser and Broom, 1990). Though deer have been the subject of intensive research over the last 30 years, relatively little is known about their physiological and behavioural responses to stress. Thus the main aim of this thesis was to investigate the activity and functioning of the HPA axis and how these change in response to acute and chronic stress in red deer.

Many aspects of red deer biology distinguish them from other more traditionai domestic ruminant species (e.g. sheep, cattle) and as such necessitate the development of species specific animal husbandry techniques. Chapter one describes some of the unique features of the biology of deer, provides a background to the development of deer farming in New Zealand and highlights some of the animal welfare issues relevant to the farming of this species.

1.2 THE BIOLOGY OF DEER

1.2.1 Classification of deer

The Family Cervidae (antlered artiodactyls) belongs to the order Artiodactyla (even-toed ungulates) and the suborder Pecora (advanced ruminants). Pecora, also termed Ruminanta, is the largest suborder of the Artiodactyla and contains the Families Bovidae (cattle, sheep, goats, antelope and gazelle), Tragulidae (chevrotains), Antilocapridae (pronghorn), Giraffidae (giraffes), Moschidae (musk deer) and the Cervidae (true deer) (Janis & Scott 1987). The Cervidae are distinguished from the other even-toed ungulates by the following features: i) they are true ruminants with a four chambered stomach, ii) they lack a gall bladder, iii) they retain rudiments of the first 2 phalanges of the lateral digits, iv) their molars bear crescentic ridges of enamel on their crowns, v) they have a cotyledonary placenta, and vi) they have fenestrated lachrymal bullae (Young 1962). However, the main distinguishing feature of the family Cervidae is the presence of antlers on the males, with the exception of reindeer and caribou (where both male and female deer develop antlers), and the Chinese water deer (*Hydropotes inermis*) where the males develop tusks (extended upper caninies) instead of antlers. The first Cervidae appeared in the fossil record in Eurasia during the Miocene and early Pliocene epoch, some 20 million years ago (Loudon & Brinklow 1992, Putman 1988). Many of the common features of Cervidae physiology and general biology reflect their origins in the forest or woodland habitats of the temperate regions of the Northern Hemisphere. However, the 40 or so species living today have diversified to occupy such a wide range of different environments that patterns of habitat use, diet, social organisation and behaviour are highly variable, as each species has adapted to its particular environment (Putman 1988). This adaptive variation even extends to different populations of the same species when they occur in different environments, making the classification of the Cervidae rather complex. The number of species and subspecies currently recognised vary considerably depending on which classification source one uses (e.g. Harrington 1985, Whitehead 1993). Whitehead (1993) recently proposed that the Family Cervidae comprised of 6 subfamilies (see also Figure 1.1):

- Hydropotinae (represented by a single species: *Hydropotes inermis,* the Chinese water deer).
    - Muntiacinae (muntjacs and tufted deer).
    - Cervinae (Eurasian deer such as red deer).
- Odocoileinae (roe deer and the New World deer species such as marsh deer, huemul, guemal, brocket deer, white-tailed deer, black-tailed deer, pampus deer and pudu).
    - Rangiferinae (reindeer, caribou).
    - Alcinae (elk, moose).

The most diverse is the subfamily Cervinae which contains the greatest number of genera and species. The four genera which make up the Cervinae are the:

- *Axis* (comprising 1 species in the sub-genus *Axis*, the axis deer (*A. axis*) and 3 species in the sub-genus *Hyelaphus*, the hog deer (*A. porcinus*), bawean deer (*A. kuhlii*) and calamain deer (*A. calaminianensis*)).
- *Dama* (comprising 1 species, the fallow deer (*Dama dama*)).
- *Elaphurus* (comprising 1 species, the Père David's deer (*Elaphurus davidianus*)).
- *Cervus* (the largest and most widespread genera, comprising 1 species in the sub-genus *Panolia*, the Eld's deer (*C. eldi*), 1 species in the sub-genus *Przewalskium*, the Thorold's deer (*C. albirostris*), 1 species in the sub-genus *Rucervus*, the swamp deer (*C. duvauceli*), 3 species in the sub-genus *Rusa*, the sambar (*C. unicolor*), rusa deer (*C. timorensis*), philippine spotted deer (*C. alfredi*), 1 species in the sub-genus *Sika*, the sika deer (*C. nippon*), 1 probably extinct species in the sub-genus *Thaocervus*, Schomburgk's deer (*C. schomburgki*) and 2 species in the sub-genus *Cervus*, the wapiti (*C. canadensis*) and red deer (*C. elaphus*).

The North American wapiti (*C. canadensis*) is also considered by many to be another subspecies of red deer (Ellerman & Morrison-Scott 1951), however others (Clutton-Brock *et al.* 1982, Whitehead 1993) have argued they are a distinct species, usually on the grounds of size, colouring and vocalisations.

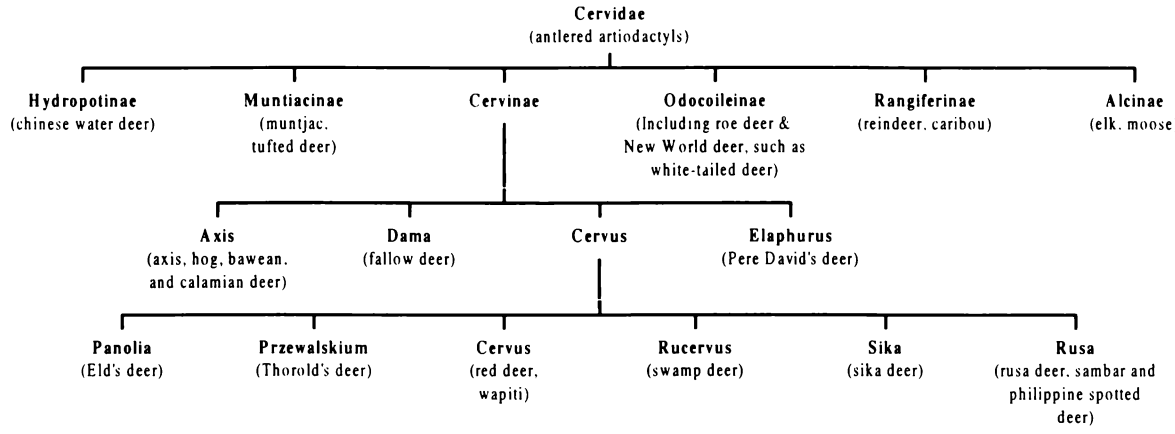


Figure 1.1 Classification of the family Cervidae (adapted from Whitehead 1993).

1.3 BIOLOGY AND DISTRIBUTION OF RED DEER (*C. elaphus* Linnaeus, 1758)

Red deer are medium to large deer with a relatively coarse coat which is a rich, reddish brown colour in summer, which becomes a greyish brown in winter. Natural populations of red deer occur widely through Europe and Asia. Most of the habitat in Europe is composed of deciduous forest, however some populations have adapted in various ways to more open habitats, such as the moors of Scotland. The large range in habitats and geographical isolation has lead to considerable phenotypic variation, mostly in size and weight. For example, the males of the subspecies of European red deer (*C. e. hippelaphus*), can reach weights of 300 kg, while the males of red deer from the island of Corsica (*C. e. corsicanus*) may weigh as little as 100 kg (Putman 1988). A number of different populations of red deer have been accorded subspecies status due to differences in size, antler shape, behaviour and vocalisations (Table 1.1).

Classification	Geographical distribution
C. e. elaphus	Sweden
C. e. hippelaphus	Europe
C. e. corsicanus	Corsica and Sardinia
C. e. wallichi	Tibet and mid-Asia
C. e. barbarus	North Africa
C. e. hanglu	Kashmir
C. e. maral	Turkey and Iran
C. e. yarkandensis	Chinese Turkestan
C. e. bactrianus,	Uzbekistan
C. e. atlanticus	West coast of Norway
C. e. scoticus	Scotland
C. e. hispanicus	Spain and Portugal

Table 1.1 The classification and natural distribution of red deer sub-species (Whitehead 1993).

1.3.1 Social behaviour

Red deer are essentially gregarious in nature (considered to be an adaptation against predation (Clutton-Brock *et al.* 1982)), although in the wild, social structure and behaviour are also strongly influenced by the type of environment which the deer inhabit (Putman 1988). In dense woodland, red deer operate individually but congregate in open areas. In open habitats such as the Isle of Rhum, off the coast of Scotland, female red deer associate with their dams forming relatively permanent matrilineal aggregations including the young of both sexes (Clutton-Brock *et al.* 1982). Males disperse from the females at weaning and form bachelor herds (4-7 stags of similar ages), for most of the year except during the rut when they attach themselves to a group of hinds and defend a harem (Clutton-Brock *et al.* 1982).

Dominance hierarchies are a feature of both male and female groupings, with a wide range of agonistic behaviours used in the establishment and maintenance of these hierarchies (reviewed by Clutton-Brock *et al.* 1982). Among hinds, the most common forms of agonistic interactions are threats such as laying the ears back and the displacement of opponents by walking steadily towards them. More

intensive interactions include kicking with one or both front legs, rearing up on hind legs and slapping at each other with the front legs (boxing), and biting or biting threats. An escalation in aggression is frequently followed by a chase or an attempt to butt the opponent (Clutton-Brock *et al.* 1982). Agonistic interactions between males involve similar activities during the velvet growing season (Haigh and Hudson 1993). However, differences are apparent during the period of hard antler with antlers become the primary means of offence and defence. Animals with the largest body size and antlers tend to have the highest social ranking.

During calving, hinds move away from their matriarchal groups to give birth (Darling 1937). New-born calves exhibit behaviour characteristic of "lying-out species", remaining hidden for the first 2-3 weeks of life (Lent 1974). For the first few days of this period, the calf freezes in response to disturbances (e.g. an approaching human) (Kelly & Drew 1976). Thereafter, a strong flight response to threatening stimuli is shown. By about 3 weeks of age, the calf begins to follow its mother. It is at this time that the mother returns to the matriarchal group with the young and the foundations for leader-follower and herding behaviour typical of adult deer are established (Matthews 1994).

1.3.2 Seasonality

Red deer evolved in environments with pronounced seasonal changes in climate and food supply. To survive these conditions red deer have evolved a range of behavioural and physiological adaptations, including marked seasonal changes in reproductive function and sexual behaviour (Lincoln 1971, Lincoln & Kay 1979, Suttie *et al.* 1992), voluntary food intake (VFI) (Loudon *et al.* 1989, Loudon 1994), and growth rate (Kay *et al.* 1980). The majority of species in temperate and cold climates exhibit changes in reproductive activity in response to annual changes in day length (photoperiod) which is consistent from year to year within a given latitude. Other environmental cues such as temperature, rainfall and food availability, exhibit annual variations from year to year which make them poor predictive cues for breeding (Bronson 1985).

1.3.2.1 Photoperiodism

The annual change in photoperiod has been shown to provide the key proximate environmental cue for the entrainment of annual cycles in deer. The manipulation of photoperiod or the administration of melatonin results in a shift in the timing of the breeding cycle, the seasonal cycle in appetite and the moult cycle (Adam & Atkinson 1984, Adam *et al.* 1986, Milne *et al.* 1990, Loudon & Brinklow 1992, Loudon 1994). The pineal hormone melatonin, which is secreted maximally during darkness, relays to the deer the length of the night and hence the season of the year providing the main link between the external environment and the endocrine system. In this respect, red deer are similar to other seasonal mammals (reviewed in Lincoln 1985, 1992, Loudon & Brinklow 1992). Removal or denervation of the pineal gland in red deer does not eliminate the annual cyclicity of reproduction (Lincoln 1985). The cycles persist but are often irregular or out of phase with the normal cycle (Lincoln 1985), indicating the existence in cervids of an underlying endogenous annual rhythm which is entrained (synchronised) by the photoperiod.

Seasonal reproductive changes in red deer are mediated through circannual variation in endogenous gonadotrophin-releasing hormone (GnRH) release from the hypothalamus. This decapeptide controls the reproductive axis by stimulating the secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland (Lincoln 1985). The gradual change from long days to short days following the summer solstice causes an increase in the duration of nocturnal melatonin secretion, which in turn induces an increase in pulsatile GnRH secretion (Figure 1.2). The mechanism by which melatonin acts within the brain to influence GnRH release is still unresolved, but it is assumed to influence the neural systems within the hypothalamus which regulate pulsatile secretion of GnRH.

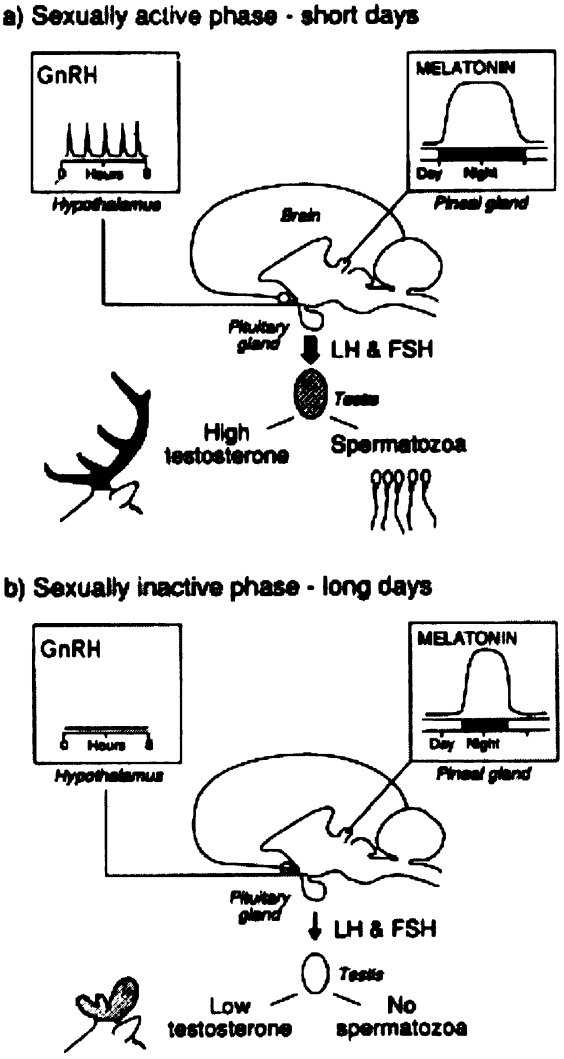


Figure 1.2 Diagram of the endocrine control of seasonal reproduction in red deer stags: (a) sexually active phase (short days) induced by high frequency GnRH secretion from the neurosecretory cells in the hypothalamus that stimulates LH and FSH secretion from the pituitary gland and thus gonadal activity. Short days and long nights result in increased melatonin secretion from the pineal gland which acts within the brain to affect GnRH secretion, (b) sexually inactive phase, long days and short nights result in reduced secretion of melatonin which switches off GnRH secretion (adapted from Lincoln 1992).

Seasonal variations in plasma LH and FSH has been demonstrated in red deer (Lincoln & Kay 1979, Kelly *et al.* 1982, Suttie *et al.* 1984, 1992). The pulsatile secretion of LH is of low amplitude and frequency during the non-breeding season (early summer) and of high amplitude and frequency leading up to the onset of the breeding season in autumn (Lincoln & Kay 1979). The effect of season on basal and GnRH-induced LH secretion is believed to involve both a steroid-independent mechanism (the seasonal rhythm in GnRH secretion), and a second steroid-

dependent mechanism (changes in the sensitivity of pituitary LH secretion to the negative feedback effect of ovarian or testicular steroids, Karsch *et al.* 1984). The latter is believed to amplify the effects both temporally and in magnitude of the steroid-independent mechanism (Lincoln & Short 1980, Meikle & Fisher 1996)

1.3.2.2 Annual cycle of reproduction in female red deer

Red deer hinds reach puberty at 15 months of age provided a minimum liveweight of 60-70 kg has been attained (Kelly & Moore 1977). Breeding in red deer is timed so that the most energetically demanding phase of the reproductive cycle for the female (late pregnancy and lactation), coincides with periods of maximum food abundance (Loudon & Jabbour 1994). Mating occurs in autumn (April, Southern Hemisphere) and calving in summer (late November-December), following a 233 day gestation period (Kelly *et al.* 1982). Conception rates following natural mating to spontaneous oestrus are in the order of 80% or more (Fennessy *et al.* 1990). Female red deer are polyoestrus with nonpregnant hinds exhibiting periods of oestrous cyclicity starting in autumn with 5-8 oestrus cycles of 18-20 days (Loudon *et al.* 1989, Meikle & Fisher 1996). A extended period of anoestrum occurs in spring and summer characterised by low levels of progesterone secretion indicating complete ovulatory arrest (Guinness *et al.* 1971, Kelly & Moore 1977, Asher *et al.*1997). Figure 1.3 illustrates the annual reproductive changes in relation to photoperiod.

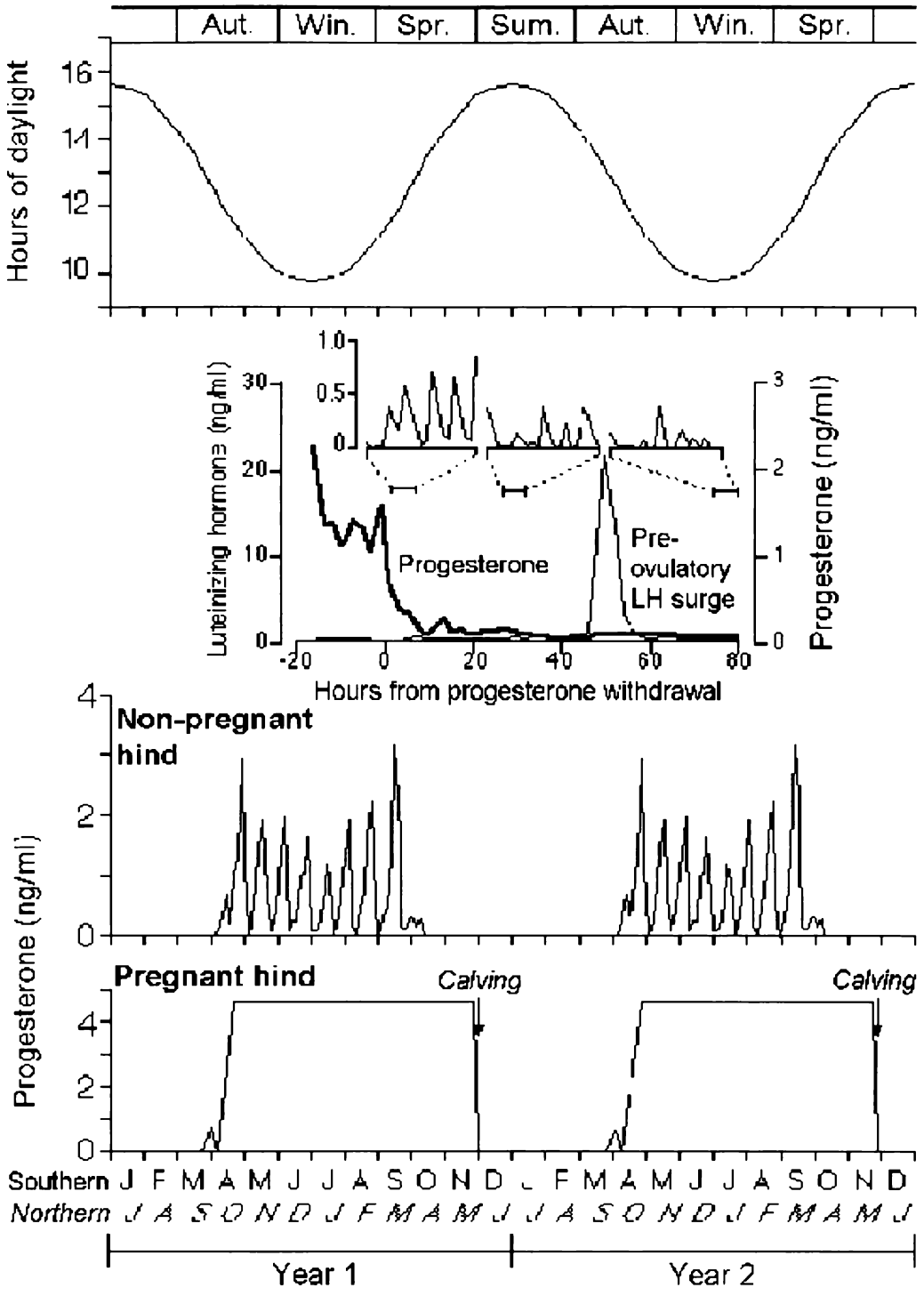


Figure 1.3 Annual reproductive cycle of female red deer in relation to photoperiod and season (from Berg 1997).

1.3.2.3 Annual cycle of reproduction in male red deer

Red deer stags are polygamous and the dominant male mates with several females by monopolising a harem during the rut (Lincoln 1971). Stags attain puberty, as defined by the onset of androgenesis, between 9-15 months of age (Lincoln 1971b). Sexual maturity, however, is not complete until approximately 7 years of age, when the stag assumes an effective role in the reproduction of the population in the wild. The breeding cycle of the stag is timed to coincide with the availability of females in oestrus (Lincoln 1992). The breeding season occurs during the decreasing daylength of late summer and autumn, hence red deer are termed "short day breeders". The rut is a period of hypersexual activity during the breeding season (Lincoln & Guinness 1973). The rut lasts 4 to 6 weeks with most conceptions occurring within a 3-week period (Lincoln & Guinness 1973). However, the stag remains fertile throughout the entire breeding season (5 months) which is followed by a 3 month period in spring/summer of complete reproductive quiescence.

The seasonal cycle in reproductive function in the stag is associated with marked changes in the activity of the hypothalamic-pituitary gonadal (HPG) axis and associated changes in testicular development, spermatogenesis, androgenesis, and the development of secondary sexual characteristics (e.g. inducing the hard antler phase, neck muscle hypertrophy, and the overt sexual and aggressive activities characteristic of the rut (Asher *et al.* 1994)). Marked seasonal changes in testicular function are controlled primarily by these changes in LH secretion. Testicular development, spermatogenesis, androgenesis, and the development of secondary sexual characteristics as well as the expression of overt sexual and aggressive activities increase leading up to the rut. By the onset of the rut, stags are fully fertile producing large numbers of viable spermatozoa and high concentrations of circulating testosterone (Figure 1.4) (Suttie *et al.* 1992). Stags remain fertile throughout the winter, with the testes secreting modest levels of testosterone.

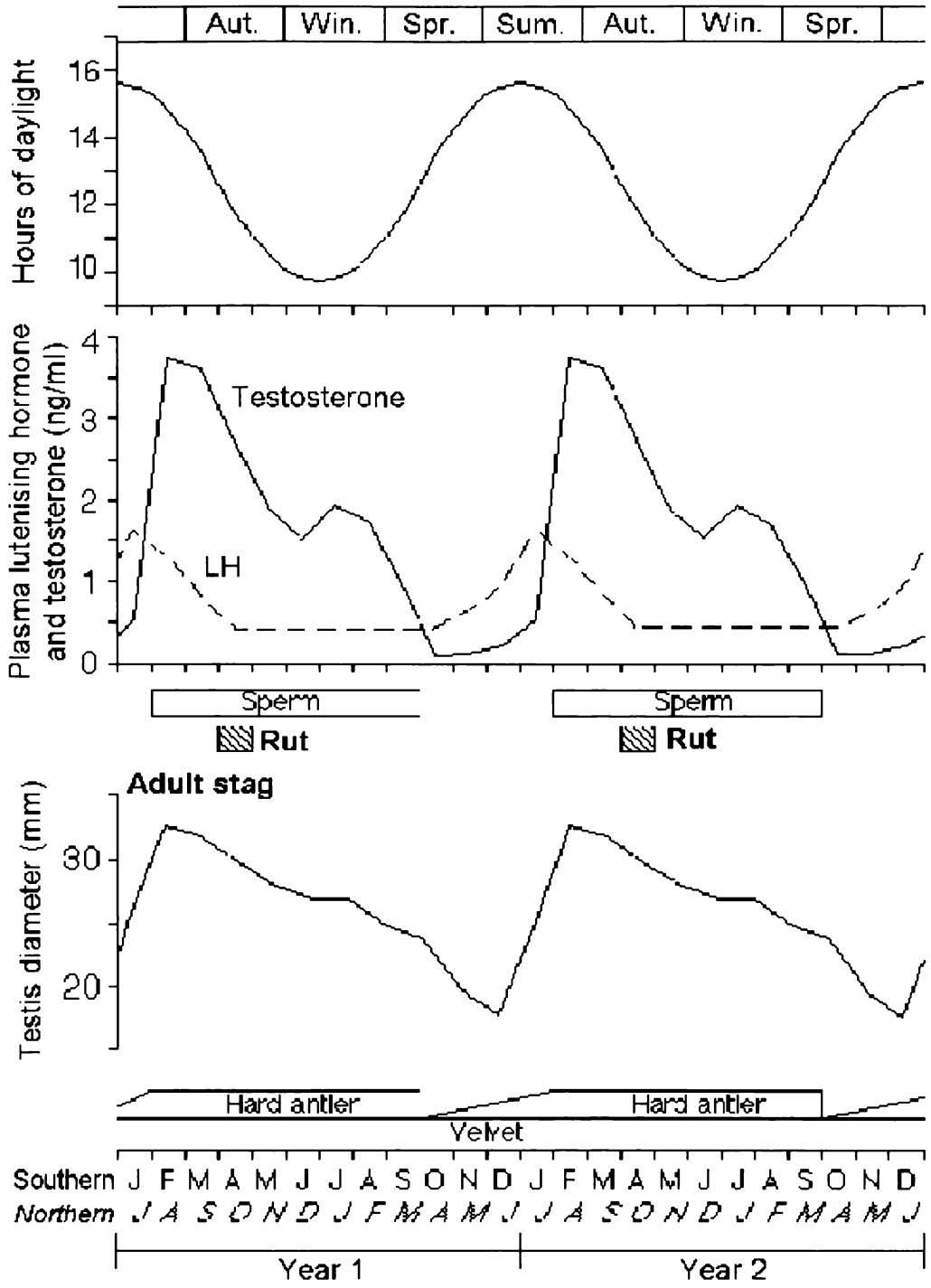


Figure 1.4 Annual reproductive cycle of male red deer in relation to photoperiod and season (from Berg 1997).

However, towards the start of spring, reproductive function begins to decline with LH secretion diminishing and testosterone concentrations reaching very low levels

as the testes regress in size. By early summer, HPG axis activity has ceased and the stags are effectively infertile and behave in a similar manner to castrates (Lincoln 1985, 1992). The animals remain infertile for about two months during the period of velvet antler growth, gradually regaining fertility towards the end of summer (Suttie *et al.* 1992).

1.3.2.3.1 Secondary sexual characteristics

The antler cycle is linked closely to the cycle in reproductive function, with antlers cast during the spring in response to regression of the testes and a marked decline in testosterone secretion (Lincoln 1985, 1992). New antler growth (velvet) occurs during the phase of reproductive quiescence when testosterone secretion is negligible (Suttie *et al.* 1992). As testicular activity and testosterone concentration increase in late summer and early autumn, the antler mineralises rapidly and eventually the soft velvet layer is stripped off. The hard antlers are retained through autumn and winter while circulating levels of testosterone are maintained.

Increasing secretion of testosterone in late summer/early autumn also causes hypertrophy of the neck muscles in red deer stags resulting in a massive increase in neck muscle mass by the start of the rut (Lincoln 1971). However, neck girth decreases with testicular regression and antler casting. Overt sexual activities, increased aggression (Suttie 1985), vocalisations and distinctive odours related to rutting activity are also associated with annual changes in testosterone secretion and can be modified by castration (Lincoln *et al.* 1972)

1.3.2.4 Annual cycle in growth and metabolism.

In addition to annual cycles in red deer reproduction, there are also pronounced cycles in voluntary food intake (Loudon *et al.* 1989, Loudon 1994), metabolism (Simpson *et al.* 1977) and growth (Kay *et al.* 1980). In both sexes the endogenous seasonal growth and appetite cycles are associated with the annual cycle of energy expenditure resulting from reproduction (Loudon & Brinklow 1992). In males, rapid liveweight gains occur during spring and summer with increased deposition of subcutaneous and depot fat as well as increased neck muscle mass (Lincoln *et*

al. 1972). The seasonal peak in VFI and liveweight occurs just prior to the onset of the rut (Fennessy *et al.* 1981). However, during the rut stags reduce the time spent grazing as they concentrate their activity on gathering, guarding and mating a harem of hinds. The resultant negative energy balance leads to rapid mobilisation of fat reserves, some catabolism of muscle, (though muscle loss is relatively small compared to the loss of fat (Wallace and Davies 1985)), and changes in the protein:water ratios in muscle (Jopson 1993). Stags regain very little of the lost weight over the winter period following the rut, even though their levels of grazing activity return to normal (Fennessy 1982). It is not until spring that the growth and fat deposition cycle starts over again.

Similarly, in females, the seasonal cycle of VFI and live weight gain and loss resembles that of the stag, at least in the growing hind, with the exception being the period of weight loss over the autumn rut is not as dramatic and the amplitude of the entire cycle is not as great as in the male. The endogenous seasonal cycle of VFI and live weight peaks in mid summer is coincident with the normal timing of peak lactation.

1.4 FARMING OF RED DEER IN NEW ZEALAND

The modern farming of red deer is relatively recent and owes its origins in New Zealand to a large, well established population of feral deer, and a flourishing export industry in feral game meat (Drew 1996). Red deer were introduced into New Zealand by early European settlers for the purposes of recreational hunting. Importation of red deer from the United Kingdom started during the 1850's (Anderson 1978) and by 1923 approximately 1000 red deer had been liberated into the wild on all three of New Zealand's main islands. With ample food, protection from hunting and an absence of natural predators feral red deer thrived, inhabiting a vast topographical range from alpine areas to coastal lowlands, and a range of habitat types including native and exotic forests, native scrublands and native grasslands (King 1990, Drew 1996).

By the 1900's, control of the feral population became necessary to prevent

damage to native and exotic forests and to reduce competition with sheep and cattle pastoral farming as well as the widespread damage of erosion prone land (Drew 1996). In 1931, the Government of New Zealand moved to bring the situation under control by introducing a deer eradication program. In the years that followed, approximately 100,000 deer were shot annually, with most carcasses left to rot (Wallis 1993). However, by the late 1950's the commercial exploitation of feral deer had begun with exports of feral venison to Germany for the game food market. Hunting pressure increased rapidly as the value of feral venison exports increased resulting in a decline in wild deer populations.

Pastoral based farming of red deer soon followed with the establishment in the early 1970's of the first commercial deer farms in New Zealand. The emphasis then shifted from the killing of the wild stock, to live capture to provide breeding farm stock. By 1985, there were nearly 400,000 red deer on farms within New Zealand with at least 60,000 caught from the wild (King 1990). The most recent survey of the New Zealand deer industry was conducted in 1996 (Statistics New Zealand 1996). In this survey total farmed deer numbers had increased to 1.5 million, with 4,000 deer farms carrying on average approximately 400 head. The industry exports approximately 94% of its total annual product output, which earned $229 million in 1996. Venison and velvet antler accounted for 66% and 25 % of this revenue, respectively (Statistics New Zealand 1996). Current exports for the year ended June 1998 have been estimated at $185 million (N.Z. Deer Farming Annual 1998).

Despite selective breeding for good temperament, farmed red deer still maintain much of the flighty nature characteristic of their wild conspecifics, exhibiting behavioural and physiological reactivity to both acute (e.g. handling, Carragher *et al.* 1997, Grigor *et al.* 1999; transport, Waas *et al.* 1997, 1999) and chronic stressors (e.g. indoor housing, Goddard *et al.* 1994, Hanlon *et al.* 1994, Pollard & Littlejohn 1998; social stress, Hanlon *et al.* 1995). Exposure to stress has also been linked in red deer to poor reproductive performance (Yerex & Spiers1987), and an increased susceptibility to infectious diseases (Griffin & Thomson 1998). As a possible consequence of their relatively recent domestication, deer may have a greater susceptibility to stress related diseases than more traditional livestock (Griffin 1989,

Griffin & Thomson 1998)

1.5 Welfare Issues in Deer Farming

Attempts to reach a consensus as to the definition of animal welfare have so far failed (Rushen & Passile 1992). Fraser & Broom (1990) stated simply that good welfare of an animal depends on the absence of suffering. Welfare can also be regarded as ideal when the physical and behavioural needs of the animal have been met. That is, when animals are free from: thirst, hunger and malnutrition; physical discomfort; pain, injury and disease; fear and distress; and are free to express normal behaviour (Farm Animal Welfare Council 1993). Increased awareness of welfare related issues in the farming of deer can be attributed to public empathy with deer, their relatively recent domestication (combined with their flighty nature) and potential susceptibility to stress related pathologies. This may mean that many practices acceptable for other domesticated species may have the potential to be detrimental to the welfare of deer (Goddard & Matthews 1994).

Early concerns were raised about the live capture of wild deer by such techniques as netting from helicopters. Capture of wild deer proved to be extremely distressing to the animal, with early experiments demonstrating gross physiological changes associated with post-capture myopathy, followed in severe cases by death within days or even hours of capture (McAllum 1985). The effect of post-capture management of wild caught animals and their adaptation to intensive farming practices has also received some attention (Griffin *et al.* 1991, Goddard *et al.* 1994a, b, 1996).

As most animals are now farm bred in New Zealand, the emphasis of research is shifting to the effects of farm management. On farms, deer are regularly handled in yards for veterinary practices such as drenching, vaccination and velvet antler removal, as well as the general movement between pastures. Previous studies have highlighted the behavioural and physiological reactivity of farmed red deer to handling (Cross *et al.* 1988, Price *et al.* 1993, Pollard & Littlejohn 1994, 1995, Pollard *et al.* 1994, Carragher *et al.* 1997, Grigor *et al.* 1999), velvet removal

(Matthews *et al.* 1992, Pollard *et al.* 1992a), disturbance at pasture (Hodgetts *et al.* 1998) and the attenuating effects of long-acting neuroleptics (Diverio *et al.* 1996a, 1996b) or the provision of visual cover (Whittington & Chamove 1995).

The effects of other current farm management procedures such as weaning of fawns from their mothers at an earlier age than would occur in the wild have also been investigated (Griffin *et al.* 1991, Pollard *et al.* 1992b, Hibma & Griffin 1994, Pollard & Pearse 1998). Weaning is considered the single most stressful event in the first year of a young deer's life (Haigh 1992) and is associated with fence pacing (Moore *et al.* 1985), immunosuppression (Griffin *et al.* 1991) and an increased susceptibly to disease (Mackintosh & Henderson 1984), particularly if weaning is associated with inclement weather conditions (Griffin *et al.* 1991).

Dominance hierarchies are a feature of both male and female groupings which when disturbed can lead to increases in aggression. In red deer, mixing of unfamiliar animals has previously been shown to be stressful both in the short term (Pollard & Littlejohn 1998) and when mixing is repeated over time (Hanlon *et al.*1995). Mixing of social groups is also thought to contribute to pre-slaughter stress in farmed deer (Kay *et al.* 1981).

Indoor wintering of deer is common in Europe and is becoming more popular in colder regions of New Zealand. Benefits include protection of animals from inclement weather, the ability to manipulate photoperiod to improve weight gains and protection of pasture from trampling (Pollard & Littlejohn 1998). Several recent studies have compared deer housed indoors with those kept at pasture and found increased levels of aggression and physical injuries such as skin damage in housed animals (Pollard & Littlejohn, 1998). Behavioural activity levels and immune responses have also been shown to be effected by indoor housing conditions (Hanlon *et al.*, 1994). The ability to access resources such as feed and water may also be compromised in some indoor housing situations

Pre-slaughter handling including transport and lairage, and their effects upon stress parameters and carcass quality have also been assessed in a number of studies (Smith & Dobson 1990, Jago *et al.* 1997, Goddard *et al.* 1997, Grigor *et al.* 1997, 1998, 1999, Waas *et al.* 1997, 1999).

In general, exposure to stress has also been linked in red deer to poor reproductive performance (Yerex & Spiers 1987), and an increased susceptibility to infectious diseases (Griffin & Thomson 1998). The continued development of management systems for farmed red deer that are safe, efficient and "welfare friendly" will depend increasingly on an understanding of fundamental aspects of deer biology and behaviour, particularly, their physiological and behavioural responses to stress.

CHAPTER 2 THE RESPONSE TO STRESS

2.1 HISTORICAL DEVELOPMENT OF THE STRESS CONCEPT

Contemporary concepts regarding homeostasis and stress can be traced back over several millennia to the Greek natural philosophers. Heracleitus (500 BC) first suggested that a static unchanged state was not the natural condition, but rather that the capacity to undergo constant change was intrinsic to all living things (cited in Griffin & Thomson 1998). Later Empedocles (500-430 BC) proposed that all matter was a harmonious mixture of elements and qualities (cited in Chrousos *et al.* 1988, Johnson *et al.* 1992). Hippocrates (460-375 BC) extended this concept to living beings, referring to health as a state of harmonious balance and disease as a state of disharmony of these elements. Hippocrates also suggested that the disharmony of disease derived from natural rather than supernatural sources and that the counterbalancing or adaptive forces were also of a natural origin, introducing the concept that "nature is the healer of disease" (cited in Chrousos & Gold 1992). Around this time, Epicurus (341-270 BC) advocated the role psychological stress plays in health and disease. He suggested that the mind could be among, or influence, these adaptive forces and that coping with emotional stressors was a way to improve the quality of life (cited in Chrousos *et al.* 1988, Chrousos & Gold 1992, Johnson *et al.* 1992).

In the early nineteenth century, the French physiologist Claude Bernard introduced the concept of the *milieu interieur*, the principle of a dynamic internal physiological equilibrium, describing its constancy as essential in an external environment typified by its variability (cited in Chrousos & Gold 1992, Griffin & Thomson 1998). The Canadian physiologist Walter Cannon later expanded this theory coining the term "homeostasis" to describe Bernard's consistency. Cannon

demonstrated the role of the sympathoadrenomedullary (SAM) system in the "fight or flight reaction" to external challenges, linking the adaptive response and catecholamine secretion, with extreme levels of activation-producing pathology (Cannon 1914). Cannon proposed that there was a critical level of stress, in terms of magnitude and duration, against which the homeostatic mechanisms fail leading to sickness and eventually death. He also demonstrated that physical and emotional disturbances were able to trigger the same physiological responses (Johnson *et al.* 1992, Terlouw *et al.* 1997).

The important role of the glucocorticoids in the response to stress was first recognised by the American physiologist Hans Seyle who reported that a wide range of noxious stimuli provoked a rise in corticosterone secretion from the adrenal cortex in rats (Seyle 1936). Debate continues as to whether it was Seyle or Cannon who first appropriated the term stress from physics. In physics, stress is precisely defined as the force applied to a spring which either elongates or compresses it, with the resulting relative change in spring length is described as strain (Yousef 1988). Seyle (1976) defined stress as it pertains to biology as the non-specific response of the body to any demand. As a consequence of the apparent generality of the physiological response, he proposed a theory which he termed the "General Adaptation Syndrome"(GAS)(Selye 1936). According to Selye the response to stress had four stages: 1) an initial alarm reaction characterised by an immediate activation of the SAM axis; 2) a stage of resistance characterised by activation of the hypothalamic-pituitary-adrenal (HPA) axis; 3) a stage of adrenal hypertrophy, gastrointestinal ulceration, and thymic and lymphoid atrophy; and 4) a final stage of exhaustion and death. Selye recognised the paradox that the physiological systems activated by stress can not only protect and restore but also damage the body.

Mason (1971) questioned the non-specificity of the physiological response implicit in the GAS model of stress. Mason pointed out that many different physical stressors also incorporated a psychological stress component, which is also a potent stimulus of the stress response. He proposed that the similarity of the physiological response might actually be a specific reaction to psychological stress, rather than a non-specific reaction for all stressors.

Current concepts regarding the specificity of the response to stress support Mason's hypothesis. Thus the response to a stressor is regarded as an activation of a complex repertoire of behavioural and physiological responses that are specific to that stressor (Mason 1968, Moberg 1985, Broom & Johnson 1993). For example, subjecting sheep to dehydration results in different patterns of neuropeptide gene expression in the hypothalamus than subjecting them to isolation (Matthews *et al.* 1993). Though the responses may be stressor specific their overall function is to produce behavioural and physiological changes directed towards maintaining homeostasis (Ewbank 1985, Moberg 1985, Johnson *et al.* 1992). Thus many of the down stream changes are similar in response to a variety of stressors.

2.2 DEFINING STRESS

2.2.1 Relevance of the stress concept

Attempts to reach a consensus as to the precise scientific definition of stress have so far failed (Rushen & Passile 1992). Toates (1995) suggested that trying to define the term stress in a scientifically rigorous way was intrinsically problematic, like defining good and evil. The complexity and apparent inconsistency in response to stress as well as the circularity of argument in many proposed definitions of stress (i.e. a stressful stimulus is something that produces a stress response and the stress response is what is produced by a stressful stimulus) have led several authors to propose that the term stress be abandoned altogether in a scientific context (Rushen 1986, Charlton 1991). Rushen (1986) stated "stress itself is one of those terms we use to shield us from our ignorance. We would be better off without it. It survives because it is a convenient term to indicate the general topic under discussion. Attempts to provide such a vague concept with a precise physiological definition engender confusion and misunderstanding".

The need to accurately define stress has also been questioned by Moberg (1985). He stated that the failure to develop a precise definition of stress is because stress

is not a single entity to be identified by a discrete response but is essentially a syndrome with no discrete aetiology, no consistent biological response, nor even a single effect on the individual. Such comments however, have failed to diminish the debate surrounding attempts to scientifically define stress and the advantages/disadvantages of particular measures of stress. Attempts at defining stress have generally focussed on the stressful stimulus, the response to that stimulus or the long-term consequences of the response.

2.2.2 Defining stress based on the stimulus

An integrative definition of stress based upon common features of stimuli has as yet not succeeded as it appears stressful stimuli are just too numerous and various to bring together in a convincing synthesis (Charlton 1991). In addition, the capacity of a particular stimulus to evoke stress depends upon factors other than its physical properties, such as the organism's control over, or familiarity with, the stimulus. This would suggest that stressors should not be defined by their intrinsic physical properties, but only by their effect upon the body. Thus, Rivier (1991a) defined stress as "any threat real or perceived which can alter homeostasis" while von Borell (1995), along similar lines, stated "stress is a broad term which implies a threat to which the body needs to adjust".

2.2.3 Defining stress based on responses

Attempts at an integrative definition of stress usually emphasise physiological or behavioural adaptive responses that occur at times when the animal encounters a stressful stimulus. Due to the pioneering research of Cannon and Selye, particular emphasis has been accorded to the activation of the SAM and HPA systems as definitive of stress. These two physiological systems are commonly involved in the reaction to stress and are activated by a diverse range of stimuli.

Definitions of stress defined in terms of activation of the SAM system include that of McCarty (1983), who proposed that a stressful stimulus was "any external event that precipitates a significant activation of the SAM system and a measurable change in behaviour". However, difficulties arise in trying to associate stress with a single physiological index such as SAM activation. In

some situations the response to stress may involve predominantly the activation of the parasympathetic nervous system (PNS). For example, parasympathetic activation is seen in the young of species that exhibit immobility responses and bradycardia when exposed to disturbing stimuli (e.g. red deer calves, Espmark & Langvatn 1985).

On the other hand, Selye (1973) considered elevations in glucocorticoids are synonymous with stress. Following this concept Allen *et al.* (1973) defined stress as "a collection of diverse stimuli which damage or potentially damage the organism and have in common an ability to stimulate adrenocorticotrophic hormone (ACTH) secretion. This results in increased glucocorticoid secretion which enables the organism to better adapt to potential or actual life threatening challenges". A number of studies have demonstrated that in acute stress situations the glucocorticoid response increases with increasing severity of the stressor (Broom 1991, Carragher *et al.* 1997). However, not all stressors induce an increase in glucocorticoids (e.g. hyperthermia in young chickens, Freeman & Manning 1984). In addition, in situations of chronic stress, glucocorticoid concentrations have been shown to be elevated, unchanged or even depressed (Jensen et al. 1996a, Rushen 1991). Glucocorticoids can also be elevated by a variety of apparently non-stressful events such as coitus, exercise and feeding and may exhibit circadian rhythms in secretion (Toates 1995). Thus it would appear that specific physiological responses such as glucocorticoid increases and activation of the SAM system may act as indicative measures of stress but changes in them are not uniquely associated with apparently "stressful" events.

There are also broad classes of behavioural changes that appear in a number of situations considered stressful. These include increased alertness and attention span, decreases in reflex time, changes in feeding and sexual behaviour as well as the development of aversions and abnormal behaviours (Chrousos *et al.* 1988, Rushen 1996). However, as with the physiological parameters, definitions of stress based on changes in behavioural parameters are not unambiguous. For example, an animal showing no obvious avoidance response to a stressful event may in fact be behaviourally inhibited due to high a high level of anxiety (Terlouw *et al.* 1997).

Recent findings suggest that corticotrophin-releasing hormone (CRH), acting as a hormone and neurotransmitter within the CNS, may play a key role in integrating the endocrine, autonomic, immunological, and behavioural responses to stress (Owens & Nemeroff 1991). Several authors have suggested that because CRH plays such a universal role in co-ordinating the response to stress, its secretion by neurones within the brain may be both necessary and sufficient to define stress (Dunn & Berridge 1990). However, given that the rate of synthesis and release of CRH can be stimulated or inhibited in different regions of the brain during stress (e.g. Makino *et al.* 1995) its value as a single measure of stress and hence definitive of stress may also be limited.

Though a convincing definition of stress has not developed from the use of these physiological and behavioural responses, they are commonly used in the assessment of stress. Thus, the activation of individual responses should be viewed as indicative of stress but are not sufficient to define stress. In order to reduce the chance of falsely ascribing stress to a situation, the assessment of stress should involve the monitoring of a wide range of stress response mechanisms in an integrated approach.

2.2.4 Defining stress based on outcomes

Another approach to defining stress is to view it as a state that arises only when adaptive mechanisms are either being chronically stretched or are acutely failing (Toates 1995). Such approaches to defining stress are linked to the concept of coping and focus on the maladaptive consequences of chronic activation of stress regulatory mechanisms. Fraser & Broom (1990) stated that "when regulatory systems are operating but are not coping with environmental conditions then the word stress should be used". The notion of coping or failing to cope and the cost to the animal in terms of energy/effort (Goddard & Matthews 1994, McEwen 1998), biological fitness (Broom and Johnson 1993) or pathology (Moberg 1985) has routinely been used in definitions of stress.

Broom and Johnson (1993) defined stress in terms of the effects upon fitness (long-term reproductive capability). They stated that "stress is an environmental

effect on an individual which overtaxes its control systems and reduces its fitness or appears likely to do so". In applying this definition, short term increases in glucocorticoids would not necessarily be seen as indicative of stress unless they have long term consequences for the animal.

Other authors have also proposed models of stress based on the long term consequences of stress. Moberg (1985) considered environmental stimuli that cause changes in both the biological functions of an animal and subsequent entry into a pre-pathological state, to be stress. In Moberg's model (Figure 2.1), the response of an animal to a stressful event starts with the recognition by the animal of a threat to homeostasis. This occurs in the central nervous system (CNS). The perception of the stressor and the organisation of a biological defence (stress response) are influenced by numerous modulating factors characteristic of the individual animal. These include the experience of the animal (Natelson *et al.* 1988), genetics (Le Neindre *et al.* 1994), age (Sapolsky *et al.* 1983), and sex (Handa *et al.* 1994). The consequence of stress, if not alleviated, is to cause the animal to enter a pre-pathological state. For example, the suppression of the immune system during stress may place an animal in a state of vulnerability. The longer the stress response persists, the longer the period of vulnerability and the greater the likelihood of the development of pathology and eventually death.

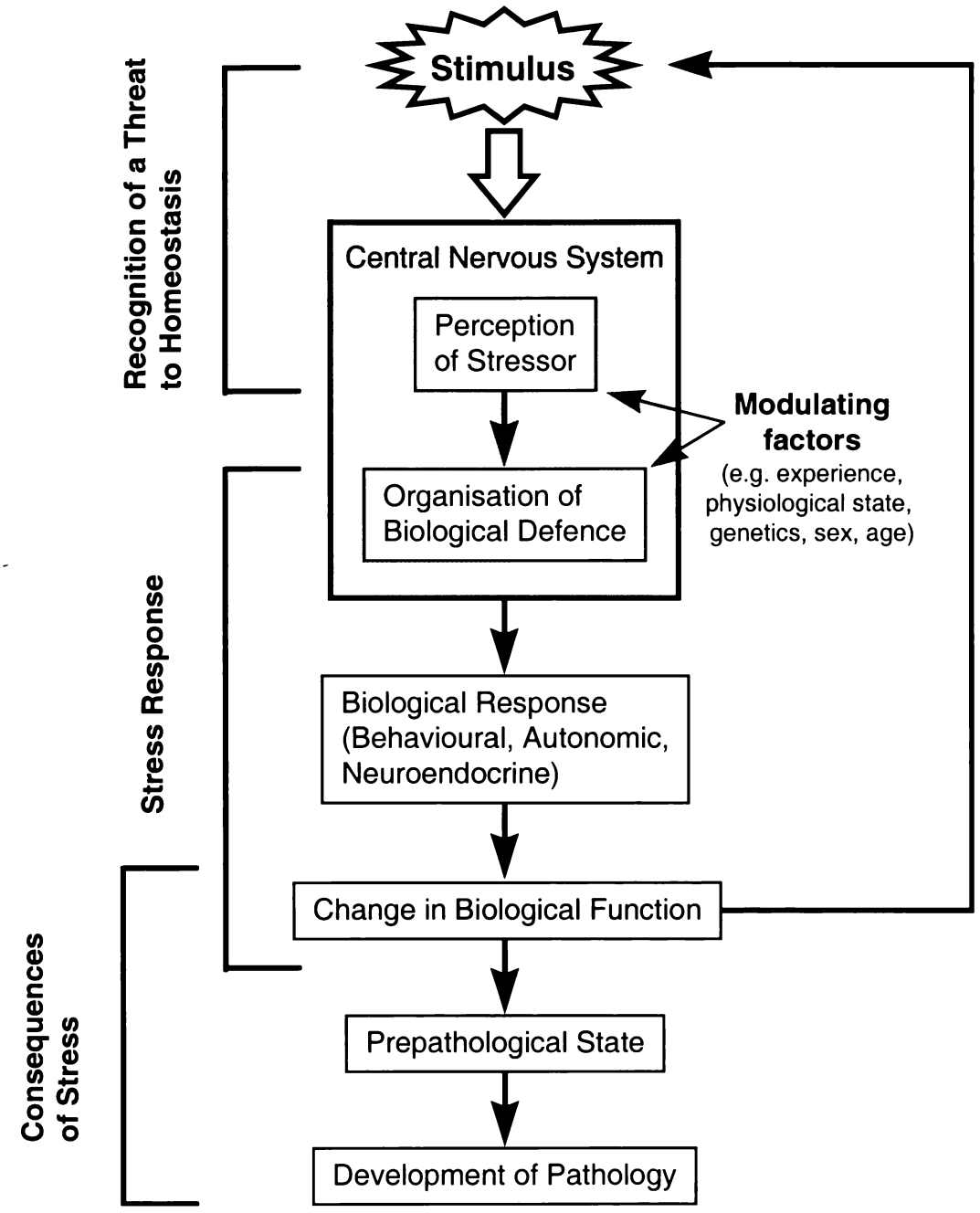


Figure 2.1 Model for the response of animals to a stressful event and examples of modulating factors that influence the perception and response to a potential stressor (adapted from Moberg 1985).

However caution must be applied in the use of such definitions. By focussing on the long-term consequences of stress, models such as those proposed by Broom and Johnson (1993) and Moberg (1985) ignore the role of stressors that do not reduce lifetime reproductive success or lead to the development of pathology. For example, in intensive farming of pigs, to achieve high piglet production (reproductive success) breeding sows are housed individually and often tethered. After long term tethering these sows exhibit overreaction of the sympathetic nervous system, hypercortisolaemia and disturbed behaviour which would appear to indicate chronic stress (Schouten & Wiegant 1997). Yet applying the model of Broom and Johnson (1993) would favour this production method over loose housing of sows where survival of piglets is reduced (Fraser & Broom 1990). Along the same lines, hens intensively farmed in battery cages exhibit many behavioural and physiological responses indicative of stress (Craig & Swanson 1994), yet battery hens have high levels of production and low rates of pathology. In contrast, hens raised in free-range conditions generally have a higher incidence of disease pathology (Fraser & Broom 1990). Thus, according to Moberg's definition battery cages could be considered a low stress alternative to free range production systems.

It is obvious from the literature that an all-inclusive theory of what constitutes stress has yet to be formulated. Indeed such a theory may not be possible or even relevant. As Moberg (1985) suggested, the failure to develop a precise definition of stress is because stress is not a single entity to be identified by a discrete response but is essentially a syndrome with no discrete aetiology, no consistent biological response, nor even a single effect on the individual. At present the identification of stress is in principle an arbitrary decision, notwithstanding, or perhaps because of the multitude of behavioural, physiological, and structural parameters that have been used as indicators of stress (Wendelaar Bonga 1997). To progress towards a better understanding of what stress is and what the implications of stress are, a detailed knowledge of the activity and function of these stress response mechanisms is required.

2.3 THE INTEGRATED RESPONSE TO STRESS

Preservation of homeostasis requires continuous behavioural, autonomic and endocrine responses to modulate/regulate the effects of a wide range of intrinsic or extrinsic stressors. Both physical and psychological stressors can activate central and peripheral responses designed to maintain homeostasis. These responses generally involve a redirection of both behaviour and energy to mitigate the effects of the stressor.

2.4 THE BEHAVIOURAL RESPONSE TO STRESS

The behavioural response to stress involves the facilitation of neural pathways mediating functions such as arousal, alertness, vigilance, cognition, and focussed attention, as well as appropriate aggression (Chrousos & Gold 1992). This occurs with a concurrent inhibition of pathways that mediate functions such as feeding and reproductive behaviour (Britton *et al.* 1982, Sirinathsinghji *et al.* 1983, Chrousos & Gold 1992). Changes are also seen in the central nervous system elements that influence the retrievability and analysis of information (e.g. selective memory enhancement, Bohus *et al.* 1983), pain perception (e.g. stress induced analgesia, Terman *et al.* 1984) and emotionality (Chrousos & Gold 1992).

2.4.1 Central co-ordination of the behavioural response to stress

The central mechanisms by which stressful situations activate behavioural responses are complex. However, many of these behavioural responses appear to be mediated by the corticotropin-releasing hormone system, which is widespread throughout the brain, and the locus ceruleus-noradrenaline (LC-NA)/autonomic (sympathetic) nervous system located in the brainstem (Chrousos & Gold 1992).

Activation of the LC-NA/sympathetic system leads to a release of noradrenaline from a dense network of neurons throughout the brain resulting in enhanced arousal and vigilance, as well as increased anxiety (Chrousos & Gold 1992). Specific brain systems activated by the LC-NA/sympathetic system during stress

include the mesocortical and mesolimbic dopamine systems (Roth *et al.* 1988) and the amygdala/hippocampus complex (Herman *et al.* 1989).

CRH systems within the CNS also play an important role in activating and co-ordinating the behavioural as well as the endocrine and autonomic responses to stress (Menzaghi *et al.* 1993, Arborelius *et al.* 1999). In the rat, central administration of CRH produces a range of anxiogenic effects that are typical of those observed during stress. These include enhancement of arousal, suppression of exploratory behaviour in a unfamiliar environment, suppression of feeding and reproductive behaviours, induction of grooming behaviour, increased conflict behaviour and enhancement of conditioned fear responses during aversive stimuli (Owens & Nemeroff 1991, Koob *et al.* 1993).

2.4.2 Behavioural measures of stress

The use of behaviour as an indicator of stress in animals is of special interest as behaviour is one of the most easily observed non-invasive measures of the response to stress. Behavioural indices of stress obtained from simple observation, have been used as an index of disturbance for various manipulations/challenges in a number of species (reviewed in Fraser & Broom 1990, Broom & Johnson 1993). Such measures include the intensity, duration and frequency of startle responses and defensive or flight reactions, non-conformance to group activity patterns and the time required to resume normal behavioural activity after stress (Matthews *et al.* 1990, Friend 1991). Increases in the frequencies of stereotypic behaviour (Pollard *et al.* 1998) and the adoption of apathetic or unresponsive behaviours (Duncan *et al.* 1989) are also indicative of stress. Features of animal interactions such as aggression and maternal behaviour (e.g. Leonard *et al.* 1994) or the dynamics of animal group structure such as spatial relationships (e.g. inter-individual distances in red deer, Diverio *et al.* 1996a) or temporal synchronisation of behaviour may also exhibit stress induced changes. A recent study by Alados *et al.* (1996) has also suggested that a loss in variability and hence complexity of behaviour can be indicative of chronic stress. They found by using fractal analysis of head lifting behaviour and feeding-

nonfeeding activity sequences in female Spanish ibex (*Capra pyrenaica*) that complexity declined in animals infected with parasites.

Emotional states of animals (e.g. fearfulness, anxiety and depression) also exhibit changes associated with stress. Though the emotionality of an animal can not be determined directly, it is often inferred by the animal's behavioural response to a novel and presumably stressful situation. For example, chronically stressed rats when placed in a novel arena (open field test) defecate more and are less active, exhibiting less exploratory behaviour of the central zone of the open field than unstressed controls (van Dijken *et al.* 1992). Increased levels of fear and/or anxiety have been inferred from a reduction in the exploration of the open arms of a elevated plus-maze, a decreased response latency in an one-way avoidance-escape task and a exaggerated immobility response to a sudden reduction in background noise level while placed in a novel environment (Liebsch *et al.* 1998). Rats selectively bred for high levels of anxiety-related behaviours will stop swimming and start floating earlier, spend significantly more time in this immobile posture and struggle less during the Porsolt forced-swim test of 'behavioural despair' than rats bred for low levels of anxiety-related behaviours (Liebsch *et al.* 1998).

A more direct way to access information about the way an animal is responding to challenges is to ask it in preference or motivation tests (reviewed in Fraser & Matthews 1997). Similar information on an animal's perception of stressors can be gauged by recording time taken by an animal to approach a situation or location where an aversive treatment has taken place (Rushen 1990). For example, Fell & Shutt (1989) reported measuring the degree of aversion of sheep to a human handler involved in the application of a stressor (mulesing) using a motivational choice open field test. This test involved placing the sheep in an arena with the handler positioned between it and a stimulus the animal would approach (a flock of sheep). By using this approach-avoidance conflict they were able to assess cognitive as well as motor responses to the presence of the handler. Such simple tests of preference or aversion, however, do not readily provide a quantitative measure of the degree of aversiveness or pleasantness of environmental events or challenges (i.e. these measures are relative, not absolute).

A more quantifiable approach has involved the use of operant techniques based on behavioural demand (Dawkins 1990). Using this procedure animals pay a price to gain access to or avoid environmental stimuli. The demand (consumption) is measured as the slope of the function relating changes in consumption to changes in price. For events that the animal has a strong need to avoid, or gain access to, the slope of the function (demand elasticity) approaches zero (e.g. food). If the demand elasticity is high then the importance the animal attaches to the event or item is low. By measuring the demand elasticity, in this case to avoid a stressor, the animal's own perception of the aversiveness of that stressor can be quantified, allowing different types of stressor to compared and their aversiveness ranked from the animal's perspective.

The use of behavioural measures of stress offers unique insight into the internal state of animals. However, as with physiological indices of stress, caution must be used in interpretation of behaviour and behavioural changes. A detailed knowledge is required on what constitutes normal behaviour for that individual under a wide variety of circumstances. The developmental causes and consequences of performing or not performing particular behaviours also needs to be known. The interpretation of behavioural changes may also vary considerably with different types of stressors and between individuals (e.g. active and passive behavioural coping strategies (Benus *et al.* 1991)), they may also simply reflect a successful adaptive response to a particular challenge. Caution must also be exercised during interpretation of preference type tests as it is often assumed that the animal is choosing in its own best interests and this may not always be the true. The pitfalls to be guarded against when using preference tests are discussed in more detail by Duncan (1992).

2.5 THE PHYSIOLOGICAL RESPONSE TO STRESS

The physiological adaptation to stressful stimuli primarily involves the provision of the energy necessary to overcome the stressor. This involves both a shift of energy substrates from storage sites (e.g. liver, adipose tissue, muscle) to the circulation, as well as appropriate cardiovascular changes including increased or decreased heart rate, blood pressure and respiration. Changes in response to stress include changes in concentrations of various plasma hormones including glucocorticoids, catecholamines, endogenous opioid peptides, sex hormones, growth hormones, thyroid hormones, vasopressin and insulin (Terlouw *et al.* 1997). Glucocorticoids, and the catecholamines, adrenaline (A), and noradrenaline (NA), act to inhibit glucose uptake, fatty acid storage, and protein synthesis at storage sites and stimulate the release of these energy substrates from muscle, adipose tissue and the liver (Munck *et al.* 1984). Simultaneously, anabolic processes such as digestion, growth, reproduction and immune function are suppressed to preserve energy that could be used to greater advantage by other systems in the response to stress. The main physiological systems involved in mediating the stress response and maintaining basal and stress-related homeostasis are the autonomic nervous system and the hypothalamic pituitary adrenal axis.

2.6 THE AUTONOMIC NERVOUS SYSTEM

The autonomic or involuntary nervous system (ANS) is one of the primary physiological systems involved in the maintenance of homeostasis and the response to stress. The ANS co-ordinates cardiovascular, respiratory, digestive, thermoregulatory, excretory, and reproductive functions (see Martini 1995). The regulation of homeostasis by the ANS is achieved via both simple reflexes based in the spinal cord which provide relatively rapid and automatic responses to stimuli, and more complex reflexes which are co-ordinated by processing centres concerned with specific visceral functions located in the medulla oblongata (e.g.

cardiac centre, respiratory centre). These medullary centres are subject to regulation by the hypothalamus, which in turn receives inputs from higher regions of the CNS including the limbic system, thalamus and cerebral cortex.

The ANS is comprised of two branches, the sympathetic and the parasympathetic divisions. Most vital organs (e.g. heart, lungs) receive innervation from both divisions of the ANS (Martini 1995). In most cases of dual innervation, the two autonomic divisions have opposing effects, thus inhibition of one division can produce effects analogous to those caused by activation of the other. For example, the rate and force of contraction of the heart, which receives both sympathetic and parasympathetic innervation, is increased during sympathetic activation and/or inhibition of parasympathetic activity (Toates 1995).

A concept of autonomic reciprocity has been developed to describe the synergistic actions of the sympathetic and parasympathetic divisions of the ANS. Autonomic reciprocity encompasses three related principles: the dual sympathetic and parasympathetic innervation of target organs, the functional antagonism between the sympathetic and parasympathetic divisions, and the reciprocal control of the two divisions so that activation of one division is associated with inhibition of the other maximising the impact of excitation (Berntson *et al.* 1991). Precise control of physiological functions by the ANS is thus achieved by regulating the spontaneous activity (autonomic tone) of these two divisions. An increase in sympathetic tone generally increases alertness, stimulates metabolism and prepares the body to deal with emergencies. On the other hand increased parasympathetic tone conserves energy and promotes anabolic processes such as digestion (Martini 1995).

2.6.1 The sympathetic division of the autonomic nervous system

Cannon (1914) was the first to note that a variety of stressors resulted in an increase in sympathetic nervous system activity and adrenal medulla output, which he termed the "fight or flight" response. Since this pioneering work it has been generally accepted that the sympathetic division of the autonomic system is primarily associated with conferring an adaptive advantage during an acute

stressor. However, prolonged activation during chronic stress may be maladaptive, resulting in the development of pathologies such as hypertension (Pickering 1997).

The proposed central site of integration for the sympathetic-adrenal medullary axis is the locus ceruleus-noradrenaline (LC-NA)/sympathetic system located in the brainstem (Chrousos & Gold 1992). This nucleus receives stimulatory input from the hypothalamic CRH system as evidenced by the increase in adrenaline and noradrenaline levels following intraventricular infusion of CRH (Koob *et al.* 1988).

Most postganglionic fibres of the sympathetic division are adrenergic releasing noradrenaline (NA) from peripheral synapses. However, postganglionic fibres which innervate sweat glands and blood vessels to skeletal muscles release acetylcholine (cholinergic), while postganglionic fibres that innervate smooth muscle in the walls of small arteries in skeletal muscles and the brain release nitric oxide promoting vasodilation (Martini 1995).

The sympathetic adrenal medullary axis is a component of the sympathetic division of the ANS most frequently associated with the fight and flight response. Preganglionic fibres (cholinergic) enter the adrenal gland where they synapse with the adrenal medulla, a modified synaptic ganglion. Following stimulation the adrenal medulla releases adrenaline and to a lesser extent, noradrenaline in to the circulation where they have an effect on organs and systems throughout the body (Figure 2.2).

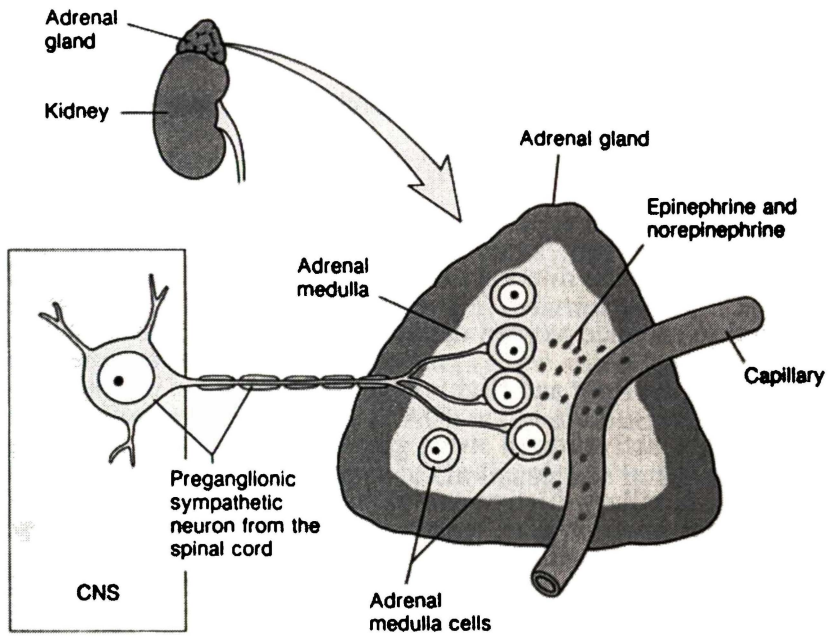


Figure 2.2 Sympathetic innervation of the adrenal medulla (Clack 1998).

2.6.2 Sympathetic activation

Activation of the sympathetic division results in the release of NA from a dense network of neurons throughout the brain resulting in enhanced arousal and vigilance, as well as increased anxiety (Chrousos & Gold 1992). Catecholamines in the circulation are also increased due to the secretion of adrenaline and noradrenaline from the adrenal medulla. In addition, increased activity of peripheral sympathetic nerve fibres produces a variety of effects in innervated target tissue. Concentrations of noradrenaline in tissue and plasma are also increased due to spill-over from these sympathetic synaptic nerve terminals.

Catecholamines have rapid and widespread actions that prepare the organism to quickly respond to stress. These actions include the rapid mobilisation of energy reserves, which provides an immediate energy source for the fight or flight response. Thus catecholamines stimulate carbohydrate metabolism glycogenolysis and gluconeogenesis in the liver, gluconeogenesis in skeletal muscles, and increase the lipolysis or conversion of triglycerides to free fatty acids and glycerol in adipose tissue. The potential for increased activity by the

organism is supported by appropriate cardiovascular changes aimed at increasing the amount of blood delivered to the skeletal muscle. These changes include increases in the rate (tachycardia) and force of contraction of the heart, cutaneous and visceral vasoconstriction with associated decreases in skeletal vascular resistance and elevations in arterial pressure. Increased respiratory rate and efficiency due in part to the dilation of bronchial musculature, tachycardia and splenic contraction enhance oxygen delivery to skeletal muscles. Other responses which contribute to the fight and flight response include the inhibition of the gastrointestinal tract, activation of sweat glands, a general elevation in muscle tone, piloerection, pupillary dilation and platelet activation (Kopin *et al.* 1988, Ruckebusch *et al.* 1991, Martini 1995).

The primary actions of noradrenaline released by synaptic knobs of sympathetic postganglionic neurons are short-lived. Post synaptic membrane effects last only a few seconds before the released noradrenaline is either removed by re-uptake and recycling at the nerve synapse, inactivated by enzymes or diffuses out of the region. The action of adrenaline and noradrenaline released from the adrenal medulla into the circulation is more widespread and of longer duration as the enzymes that metabolise catecholamines are not present in the circulation. However, catecholamines are rapidly metabolised in many tissues, including the kidneys and liver resulting in a half-life in plasma of 1-3 minutes. Enzymes such as catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) catalyse the metabolism of adrenaline and noradrenaline to metanephrine and normetanephrine and vanillylmandelic acid (VMA) (Ruckebusch *et al.*1991).

2.6.3 The parasympathetic nervous system

The parasympathetic division of the ANS includes preganglionic (first order) neurons in the brain stem and sacral segments of the spinal chord and ganglionic (second order) neurons in peripheral ganglia located within or next to target organs (Martini 1995). The parasympathetic division innervates only visceral structures serviced by cranial nerves or lying within the absominopelvic cavity. The parasympathetic division appears to produce effects antithetical to those of the sympathetic division of the ANS. Activation of the parasympathetic division

promotes quieting functions and anabolic processes. Specifically, decreased metabolic rate, heart rate and blood pressure, increased secretion by salivary and digestive glands, increased digestive tract activity, and urination and defecation (Martini 1995).

All of the pre and postganglionic fibres in the parasympathetic nervous system (PNS) release acetylcholine (ACh) (Figure 2.3) (Martini 1995). The effects of parasympathetic stimulation are usually brief (a few seconds at most) and restricted to specific organs and sites as ACh is rapidly inactivated by acetlycholinesterase within the synapse or cholinesterase in the surrounding tissue. The effect of ACh release is dependent upon the type of ACh receptor found on the postsynaptic membrane. Two different ACh receptors are present on postsynaptic membranes. Nicotinic receptors are present on ganglion cells of both the sympathetic and parasympathetic divisions. Stimulation of these receptors by ACh results in excitation via opening of membrane channels. The second type of ACh receptor is the muscarinic receptor, which is found at cholinergic neuroeffector junctions of the parasympathetic division as well as at the few cholinergic neuroeffector junctions of the sympathetic division. Activation of these receptors by ACh may produce either excitatory or inhibitory effects, depending on the nature of the enzymes activated when ACh binds to the receptor (Martini 1995).

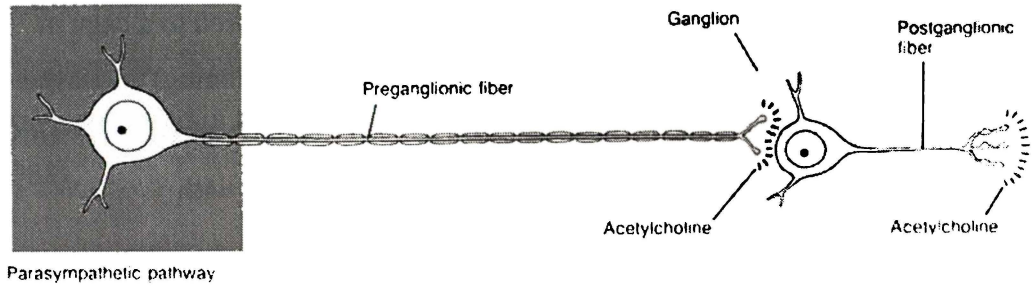


Figure 2.3 A typical neuronal pathway of the parasympathetic division of the autonomic nervous system (adapted from Clack 1998).

2.6.4 Response of the autonomic nervous system to stress

As previously stated the autonomic nervous system is one of the primary physiological systems involved in the maintenance of stress-related homeostasis. The fundamental response of the ANS to a stressor is a change in the balance of activity between the sympathetic and parasympathetic divisions. In general acute stress is associated with a significant activation of the sympathetic division of the ANS which co-ordinates responses associated with the fight or flight response. However, stress can also result in an increase in parasympathetic activity, particularly in the young of species that exhibit immobility responses and bradycardia when exposed to disturbing stimuli (e.g. red deer calves, Espmark & Langvatn 1985). Even in adult organisms, increased parasympathetic activity, evidenced by bradycardia, may be seen in certain stress situations where a passive coping strategy involving increased vigilance is adopted (Toates 1995). Korte *et al.* (1999) has also demonstrated that in chickens, active and passive coping styles could be distinguished by differences in heart rate variability (an indicator of the sympathetic and parasympathetic tone to the heart) during manual restraint, with passive animals exhibiting increased parasympathetic tone to the heart.

Given the wide range of functions regulated by both the sympathetic and parasympathetic divisions, stress related activation is often not constrained to one division. In many cases the sympathetic and parasympathetic divisions exhibit parallel activation in response to stress. For example, rats exposed to a cage in which they had previously experienced footshocks (fear conditioning) displayed simultaneous activation of the sympathetic and parasympathetic nervous systems which resulted in an attenuated tachycardia response compared with unconditioned controls (Nijsen *et al.* 1998).

Even within the sympathetic division, different types of stressors may preferentially activate either the sympathetic adrenomedullary axis or the peripheral sympathetic nerves. This is evidenced is situations of psychological stress where activation of the sympathetic adrenomedullary axis predominates and higher levels of adrenaline are found (Scheurink *et al.* 1989). In contrast, physical

activity is associated more with increased noradrenaline concentrations following peripheral sympathetic nerve activation (Scheurink *et al.* 1989). However, this generally accepted distinction may simply reflect regional differences in noradrenaline spillover and tissue metabolism. If noradrenaline spillover is determined in the whole body, noradrenaline release also increases during psychological stress (Goldstein *et al.* 1987).

The duration of the stressor and its familiarity can also influence the degree of response exhibited by the ANS. Acute exposure to stress results in significant activation of the sympathetic-adrenal medullary system. However, changes will occur in the activity and functioning of the sympathetic division following chronic or repeated exposure to the same stressful stimulus. These include increased activity of enzymes involved in catecholamine biosynthesis, increased rates of catecholamine synthesis, and elevated tissue concentrations of catecholamines indicating increased storage (Kvetnansky *et al.* 1984, McCarty *et al.* 1988). The basal levels of circulating catecholamines are increased and release of catecholamines into the circulation following repeated exposure to an identical (homotypic) stressful stimulus declines (McCarty *et al.* 1988). However, if chronically stressed animals are exposed to a novel (heterotypic) stressor, there is an exaggerated response of the sympathetic-adrenal medullary system compared to animals exposed to the same stressful stimulus for the first time (McCarty *et al.* 1988, Terlouw *et al.* 1997). The physiological responses to released catecholamines are also dependent on the receptors and receptor activated mechanisms. With repeated release of catecholamines, receptors are frequently down regulated in target tissues resulting in a reduced response (Kopin *et al.* 1988).

It would appear that a multitude of variables influence the pattern (sympathetic vs. parasympathetic) of the ANS response to stress. These include individual factors such as its stage of development and the type of coping strategy adopted as well as stressor factors such as the intensity, duration and predictability of the stressor. Thus, caution must be applied in interpreting change in ANS activity as a measure of stress. For example, stressors that produce predominately a parasympathetic activation or other stressors that favour activation of both divisions producing an

attenuated response due to the antagonism between both divisions, may be incorrectly viewed as being of low intensity.

2.6.5 Measuring the ANS response to stress.

Attempts to index the response of the ANS to stress have generally been limited to measurement of changes in activity of the sympathetic division and the quantification of catecholamines or their effects on the organism. Quantification of adrenaline and noradrenaline concentrations directly via measurement in plasma or indirectly via measurement of metabolites in urine are routinely used (Steptoe 1987, Beerda *et al.* 1996). However, caution must be used when interpreting noradrenaline levels as the concentration at the site of sampling may not reflect the systemic sympathetic activity but regional differences in noradrenaline spillover and tissue metabolism (Kopin *et al.* 1988).

The effects of sympathetic activation are also routinely used to measure the response of an organism to stress. For example, cardiovascular parameters such as blood pressure and heart rate are also routinely used as an index of the sympathetic divisions response to stress. Information on the heart rate response to stress is available for a number of species including red deer (e.g. Diverio *et al.* 1996b, Pollard *et al.* 1992, 1993, Price *et al.* 1993, Carragher *et al.* 1997, Matthews *et al.* 1994). Though heart rate is increased by stress (Blix *et al.* 1974), heart rate primarily reflects current metabolic demand (Price *et al.* 1993). Therefore, increased physical activity and basal metabolic rate are to a large extent the primary mechanisms involved in determining heart rate increases (e.g. red deer hinds, Price *et al.* 1993; white-tailed deer, Moen *et al.* 1978, Mautz & Fair 1980; sheep, Baldock *et al.* 1988; pigs, Marchant *et al.* 1997 and non human primates, Major 1998). To separate the anxiety and behavioural activity components of the heart rate response to stress, Baldock & Sibly (1990) proposed the concept of non-motor heart rate to refer to the component of the heart rate not the result of physical activity. This component is calculated by subtracting the estimated effects of behaviour from the heart rate measurement. In sheep and red deer hinds this component is increased by stress but is also effected by seasonal changes in basal metabolic rate (Price et al., 1993).

Separating the contribution of psychological and physical effects of a stressor (e.g. transport) on heart rate has also been achieved in cattle by the use of anxiolytic drugs (e.g. cholecystokinin antagonists, Jacobson & Cook 1998) or by habituation to the stressor (Cook & Jacobson 1996). However, caution must be used when interpreting results following pharmacological intervention as the efficacy, duration of effect and other potential side effects (e.g. nausea, sedative action) may in themselves contribute to measured responses and vary between species.

The analysis of short-term rhythmical oscillations in the beat to beat interval of the heart may also serve as an index of the relative activity of the sympathetic and parasympathetic divisions of the ANS (Pourges 1995). Spectral analysis allows some degree of separation of the sympathetic and vagal (parasympathetic) contributions to heart rate. High frequency (0.14-0.40 Hz) fluctuations are mediated by the parasympathetic innervation of the heart while low frequency (0.07-0.14 Hz) fluctuations are mediated by both sympathetic and parasympathetic influences (Bootsma *et al.* 1994). Using this method, changes in sympathetic and parasympathetic tone to the heart have been detected during the circadian rhythm (Furlan *et al.* 1990), moderate physical exercise (Pagani *et al.* 1988), and psychological stress (Pagani *et al.* 1991).

The effects of sympathetic activation and catecholamine release can be measured in a variety of other physiological systems. The stimulatory effect catecholamines have upon metabolism, primarily carbohydrate (glycogenolysis and gluconeogenesis) and lipid metabolism (lipolysis) can be quantified by measuring metabolites such as glucose and free fatty acids in the circulation. For example, Fernandez *et al.* (1994) demonstrated a linear correlation with glucose levels and plasma catecholamines in response to aggressive encounters in domestic pigs.

Other responses contributing to the sympathetic fight and flight response are also used in stress measurement. These include a number of features of mechanisms that contribute to the increased oxygen transport capacity of blood during stress. For example an increased respiratory rate, increased affinity of haemoglobin for oxygen, erythrocyte swelling and the increased numbers of circulating red blood cells (the result of splenic contraction) are all indicative of sympathetic activity

and catecholamine release (Nikinmaa 1992, Kock *et al.* 1987, Bateson & Bradshaw 1997). Other processes that are affected by catecholamines include the inhibition of the gastrointestinal tract, activation of sweat glands, a general elevation in muscle tone, piloerection, pupillary dilation and platelet activation (Kopin *et al.* 1988, Ruckebusch *et al.* 1991, Martini 1995).

Changes in the activity and functioning of the ANS associated with chronic stress have been implicated in the development of stress-induced hypertension (e.g. McCarty *et al.* 1987) and immunosuppression (Friedman & Irwin 1997), though other mechanisms are also involved in the development of these pathologies, e.g. the renin-angiotensin system and aldosterone in hypertension (Fraser *et al.* 1981) and the HPA axis in immunosuppression (e.g. Griffin and Thomson 1998). In humans chronic stress (caring for a spouse with a progressive dementia) enhances cardiac sympathetic activation and elevations in blood pressure following acute psychological stress, though no differences in basal activity were detected (Cacioppo *et al.* 1998).

The ANS provides a useful set of physiological parameters that respond rapidly (within seconds) to stress. Some of these parameters can be readily monitored using relatively non-invasive off the shelf technology (e.g. heart rate measurement using Polar Sports Tester, Polar Electro Oy, Kempele, Finland) and have found wide application in the assessment of acute stress in many species including red deer (e.g. Diverio *et al.* 1996b, Pollard *et al.* 1992, 1993, Price *et al.* 1993, Carragher *et al.* 1997, Matthews *et al.* 1994). However many of these responses are confounded by a multitude of variables such as increased physical activity and metabolic demand (see Price et al., 1993, Jacobson & Cook 1998) and individual and stressor factors (see previous section). Chronic stress also produces changes in ANS function that may result in pathologies such as hypertension and immunosuppression. However, the aetiologies of conditions such as hypertension and immunosuppression are complex and not solely attributable to changes in ANS function. Thus caution must be used in the interpretation of measures of ANS activity in both acute and chronic stress situations.

2.7 THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

The major endocrine response to stress involves activation of the hypothalamic-pituitary-adrenal (HPA) axis. As the name implies, the HPA axis comprises elements of the hypothalamus, anterior pituitary and adrenal cortex.

A select population of hypothalamic neurosecretory neurons in the anterior and medial-dorsal parvocellular regions of the paraventricular nucleus (PVN) function as the central integrating site for the hypothalamic pituitary adrenal axis (Herman & Cullinan 1997). Axons from these neurons project to hypophyseal portal capillaries in the external zone of the median eminence. In response to stress, these neurosecretory neurons appear to summate information from a large number of CNS sources (e.g. limbic system, cerebral cortex, brain stem), into an appropriate secretory response. This involves the release of a variety of ACTH secretagogues, the most important of which are corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), into the pituitary portal circulation. These secretagogues stimulate the release of ACTH from anterior pituitary corticotrophs, which in turn, stimulate the synthesis and secretion of glucocorticoids from the adrenal cortex (Figure 2.4).

The glucocorticoids act on cells in virtually every organ of the body, eliciting major changes in the homeostatic balance of cells by altering expression of a large number of genes (Herman 1993). Glucocorticoids increase the availability of glucose by stimulating gluconeogenesis and inhibiting glucose uptake by cells not essential to the stress response (De Kloet & Veldhuis 1985, Sapolsky 1994). They also have a major inhibitory function restraining defence reactions to stress (e.g. the immune response), that become damaging if left unchecked (Munck *et al.* 1984). Glucocorticoids also inhibit several behavioural systems and physiological processes not critical to the stress response (e.g. suppressing reproductive function, Rabin *et al.* 1988).

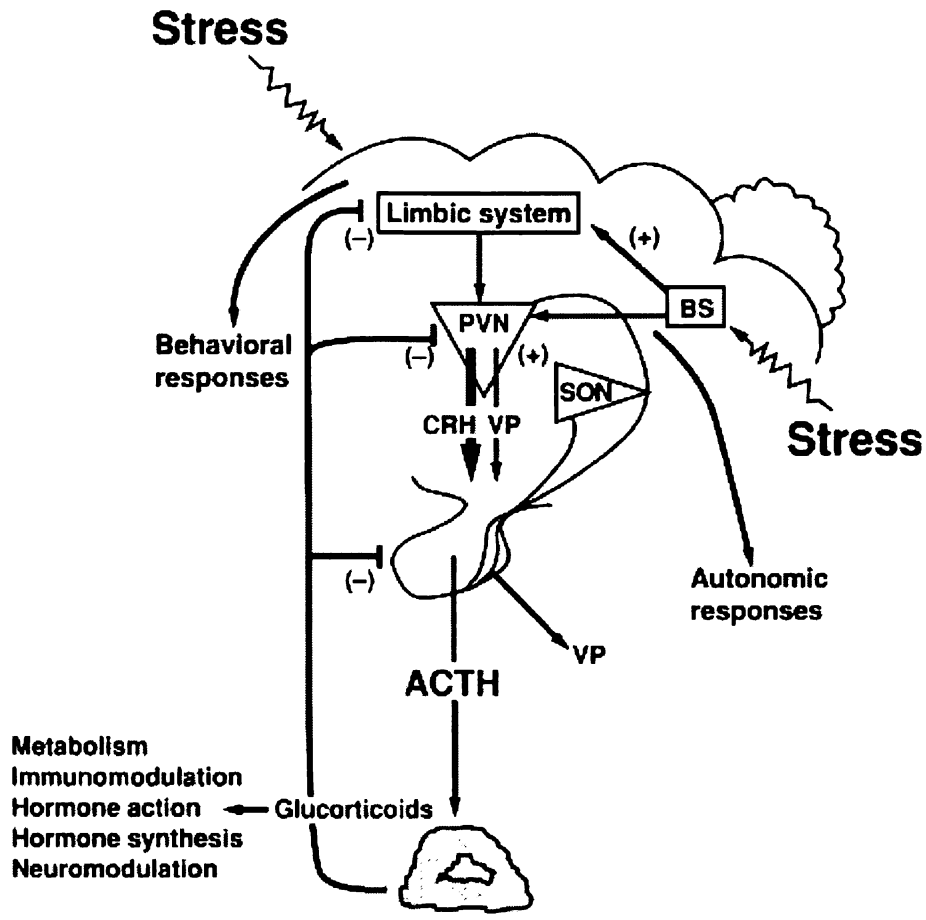


Figure 2.4 Diagram illustrating the integrated responses to stress emphasising the activation of the HPA axis, secretion of adrenocorticotrophic hormone (ACTH) and the feedback and regulatory effects of glucocorticoid. The stimulation of corticotropin-releasing hormone (CRH) and vasopressin (VP) release from the paraventricular nucleus (PVN), via inputs from the limbic system and brain stem (BS) are shown (adapted from Aguilera 1998).

Glucocorticoids also inhibit further activity of the HPA axis at various levels including suprahypothalamic levels, such as the hippocampus, as well as directly in the PVN, median eminence, pituitary and adrenal (Spinedi *et al.* 1991, reviewed in Kovaks & Sawchenko 1996, Watts 1996, Herman & Cullinan 1997, De Kloet *et al.* 1998).

2.7.1 The paraventricular nucleus of the hypothalamus

The hypothalamus is phylogenetically speaking an old region of the brain. Consequently, many of its functions are conserved among animals. The hypothalamus is a rostral extension of the brain reticular formation. Neural connections to other areas of the brain help to regulate visceral, autonomic and

behavioural functions. Many physiological functions are influenced by the hypothalamus such as growth, reproduction, lactation, basal metabolism, parameters of immune function, the state of hydration and the stress response.

The paraventricular nucleus (PVN) of the hypothalamus functions as the central focus for regulation of the HPA axis (Herman & Cullinan 1997). Parvocellular neurosecretory neurons within the PVN receive a rich afferent innervation and are believed to integrate a large variety of neuronal and hormonal stimulatory and inhibitory signals which via the HPA axis yield a physiological meaningful level of circulating glucocorticoids (Herman *et al.* 1995). The importance of this region in co-ordinating the HPA axis is evident from studies of animals with lesions of the PVN. These animals have markedly reduced portal CRH concentrations and attenuated stress induced ACTH and glucocorticoid secretion (Herman & Cullinan 1997).

2.7.1.1 Structure of the PVN

The hypothalamic PVN consists of three subdivisions (see Figure 2.5). The parvocellular division functions as the primary regulator of HPA axis activity. This division comprises a select population of neurosecretory neurons located in the anterior and medial-dorsal regions of the PVN. These neurons have axons projecting to hypophyseal portal capillaries in the external zone of the median eminence. Stimulation of these neurons results in the release of a variety of ACTH secretagogues from nerve terminals in the median eminence into the pituitary portal circulation. The ACTH secretagogues, the most important of which are CRH and AVP, are transported via the portal circulation, the short distance to the anterior pituitary where they stimulate the secretion of ACTH from corticotrophs.

Some parvocellular neurons also project to and innervate pro-opiomelanocortin containing neurons of the arcuate nucleus of the hypothalamus, as well as neurons in pain control areas of the hindbrain and spinal cord (Figure 2.5). Activation of these neurons results in CRH induced secretion of pro-opiomelanocortin derived and other opioid peptides (Nikolarakis *et al.* 1986, Burns *et al.* 1989), which can enhance analgesia (Chrousos 1992, Chrousos & Gold 1992). For example, mice

subjected to defeat in a social conflict paradigm display an analgesic response that is mediated primarily by endogenous opioids released and acting within the central nervous system (Thompson *et al.* 1988) These opioid peptides simultaneously inhibit the activity of the PVN by suppressing the secretion of CRH (Chrousos & Gold 1992, Chrousos 1992). Opioids may also be involved in the basal regulation of the HPA axis as administration of naloxone (an opioid antagonist) increases the levels of CRH, AVP and ACTH in pituitary venous blood of unperturbed horses (Alexander & Irvine 1995).

A second group of neurons located in the dorsal, medial-ventral and lateral parvocellular subdivisions also express CRH and other neuropeptides. These neurons project to the brainstem and spinal cord where they are involved in regulating the sympathoadrenal system (Saper *et al.* 1976, Swanson and Kuypers 1980, Chrousos & Gold 1992, Antoni 1993).

A third group of PVN neurons (the magnocellular division) is comprised of vasopressinergic and oxytocinergic neurons with axons projecting to the posterior pituitary through the internal zone of the median eminence (Aguilera 1998). They also have collateral terminals projecting to the portal system (Chrousos 1995). These neurons are only activated in response to osmotic stress when they, along with neurons of the supraoptic nucleus (SON), release AVP and oxytocin (OT) into the systemic circulation via the posterior pituitary (Chrousos 1995).

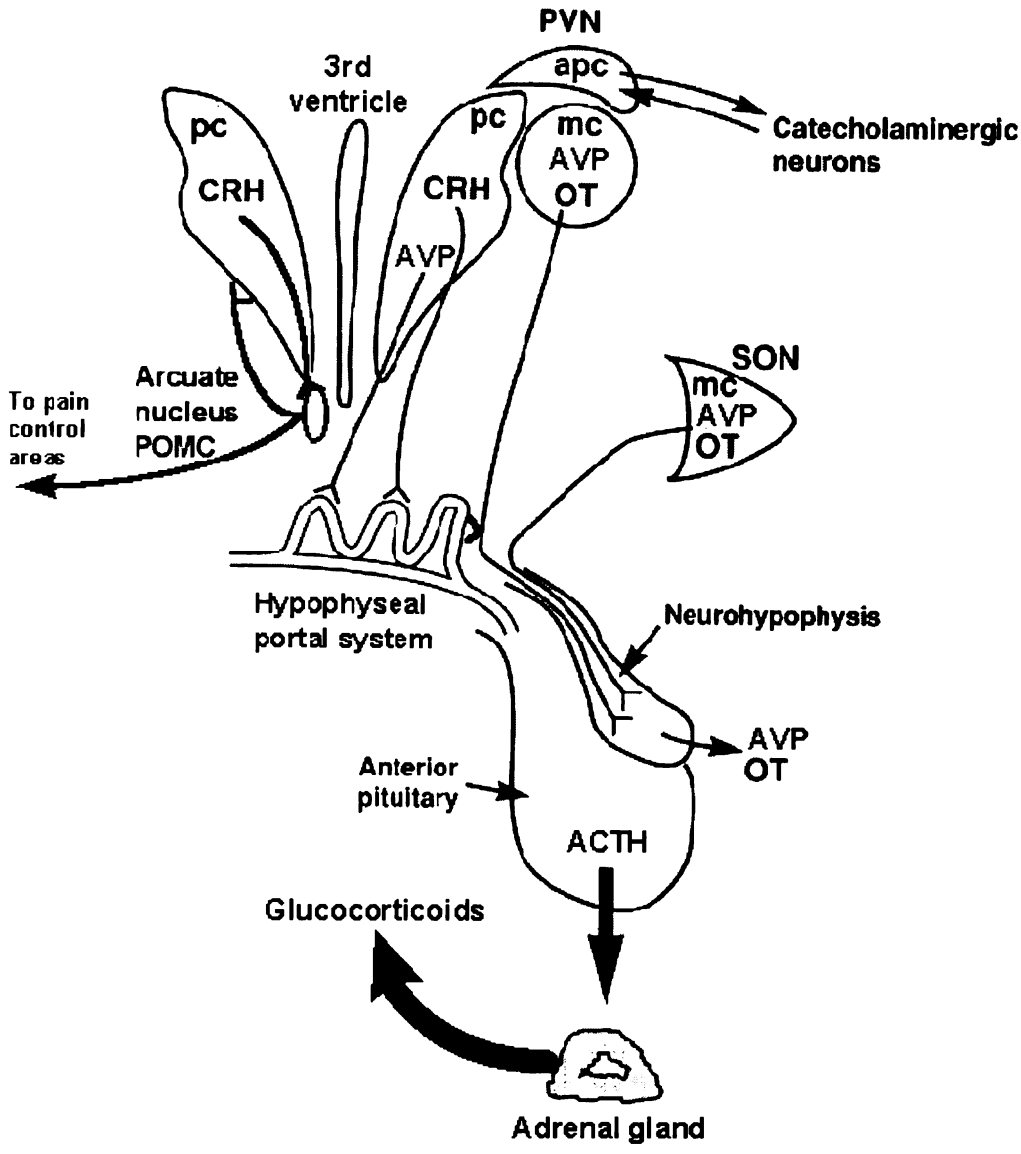


Figure 2.5 Schematic representation of the 3 subdivisions of the hypothalamic PVN. Axons from the parvocellular (pc) paraventricular nucleus (PVN) release CRH and AVP into pituitary portal capillaries stimulating the secretion of ACTH and glucocorticoids, while axons from the magnocellular (mc) division of the (PVN) and supraoptic nucleus (SON) project and transport vasopressin (VP) and oxytocin (OT) to the neurohypophysis, they also have collateral terminals in the portal system. A group of parvocellular neurons (apc) project to autonomic catecholaminergic neurons in the brain stem. The arcuate nucleus pro-opiomelanocortin (POMC) is shown, along with the mutual innervation between CRH and POMC peptide secreting neurons (adapted from Aguilera 1998, Chrousos 1995)

2.7.1.2 Regulation of the PVN

Regulation of the activity of the PVN involves a diverse set of both stimulatory and inhibitory afferents (Figure 2.6). These inputs co-ordinate the neurosecretory activity of the PVN with the characteristics of the stressor. Afferent inputs to the PVN include neuronal projections arising from ascending catecholaminergic pathways originating in the brainstem. These inputs are responsible for communicating immediate threats to survival that do not require interpretation by higher-order brain structures (see section 2.7.1.2.1). Limbic system inputs originating from the hippocampus and amygdala and relayed through the bed nucleus of the stria terminalis also modify the activity of the PVN (see section 2.7.1.2.2). These afferents are responsible for activating the PVN in response to stressors that require higher-order assembly and processing of sensory signals that do not constitute an immediate threat to physiologic homeostasis (Herman & Cullinan 1997). Other hypothalamic nuclei also project to the PVN allowing its activity to be integrated with circadian, autonomic, and behavioural regulatory mechanisms (Swanson and Kuypers 1980). Cell groups of the lamina terminalis also project to the PVN and may act as transducers of information carried by blood-borne signals (Kovács & Sawchenko 1993,1996).

An important regulator of the PVN, is its own activity and that of the rest of the HPA axis. Inhibitory mechanisms include a CRH mediated feedback loop within the PVN itself, a inhibitory feedback loop within the hypothalamus involving POMC gene-derived peptides such as ACTH and β-endorphin (Johnson *et al.* 1992). Glucocorticoids also inhibit further activity of the PVN at various levels including suprahypothalamic levels, such as the hippocampus, as well as directly in the PVN (Spinedi *et al.* 1991, reviewed in Watts 1996). The neural pathways, neurotransmitters, neuropeptides and secondary messengers involved in the regulation of PVN have been reviewed in Kovaks and Sawchenko (1996), Watts (1996) and Herman & Cullinan (1997).

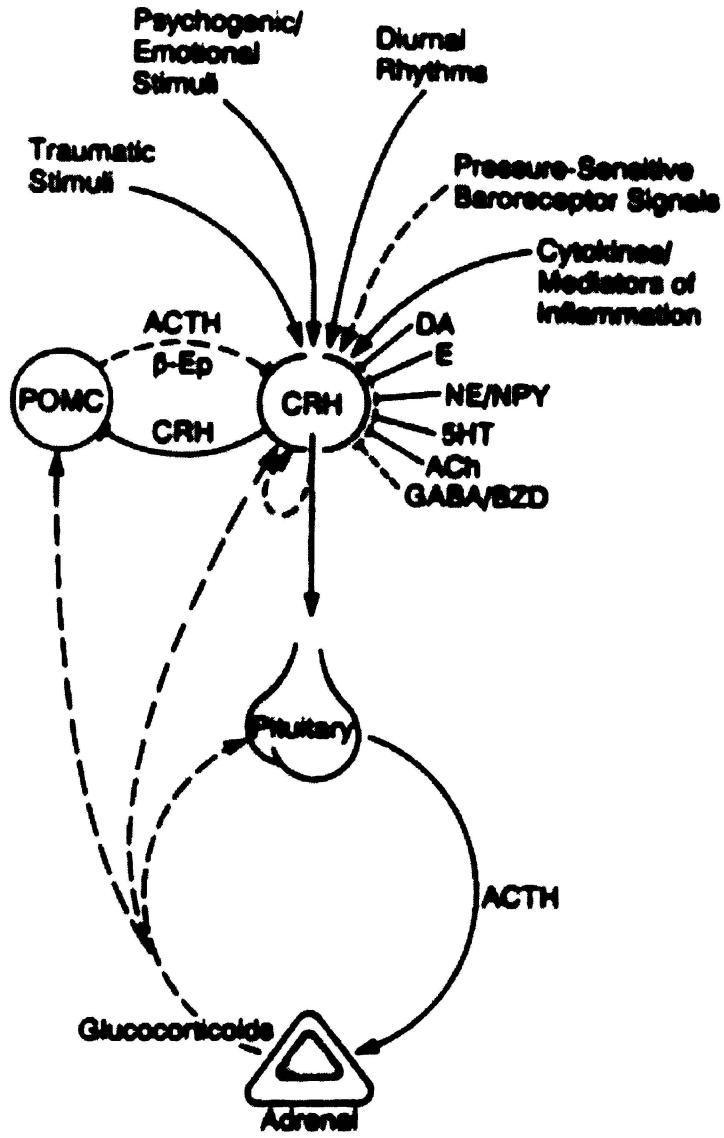


Figure 2.6 Schematic representation of the regulation of the HPA axis. The CRH system is the principal central biologic effector which facilitates a characteristic behavioural and peripheral response to stress. CRH stimulates secretion of both hypothalamic and pituitary POMC gene derived peptides, the latter resulting in glucocorticoid secretion. The activation of the CRH neurons appears to be regulated by central stimulatory and inhibitory inputs and by multiple negative feedback loops (solid lines represent stimulatory effects, broken line represent inhibitory effects). Inhibitory effects include an ultrashort CRH mediated loop, a short hypothalamic POMC gene-derived peptide loop, including both ACTH and β-endorphin, and a long glucocorticoid mediated feedback loop (Johnson *et al.* 1992).

2.7.1.2.1 Ascending catecholaminergic inputs

The HPA axis response to physiologic threats (i.e. systemic stressors which constitute a direct threat to survival), such as respiratory distress, haemorrhage, hypotension and possibly immune challenge, are rapidly relayed directly to the PVN by visceral efferent pathways originating from catecholaminergic neurons (mainly A1, A2, C1 and C2) in the brainstem (Plotsky *et al.* 1989, Ericsson *et al.* 1994, Herman & Cullinan 1997). The common attributes of these stressors are that they constitute immediate threats to survival and do not require interpretation by higher-order brain structures, enabling access to the PVN via a relatively direct pathway. For example, information on blood oxygenation is relayed from sensory elements in the carotid body or carotid sinus to the PVN by way of a single synapse with (catecholamine-containing) neurons in the nucleus of the solitary tract or ventrolateral medulla (Swanson & Sawchenko 1983). The directness of this pathway bypasses the need for cognitive processing by higher brain structures, allowing the rapid relay of an excitatory signal to the PVN. This rapid response is likely to reflect the overwhelming importance of regaining cardiovascular or respiratory homeostasis (Herman & Cullinan 1997). The excitatory effects of catecholamines on HPA activation appear to be mediated in the PVN by α-adrenoreceptors (Plotsky *et al.* 1989).

2.7.1.2.2 Limbic system inputs

In general, stressors that stimulate the HPA axis via limbic stress pathways require higher-order assembly and processing of sensory signals that do not constitute an immediate threat to physiologic homeostasis (Herman & Cullinan 1997). The function of the limbic system includes processing of memories, creation of emotional states, drives and associated behaviours (Martini 1995). Novel or threatening stimuli that act upon the HPA axis via limbic circuits requires comparison with previous experience before categorisation as stressful or unstressful. Limbic circuits are capable of augmenting or diminishing the resultant HPA response, depending on prior experience or the ongoing level of activation (Herman & Cullinan 1997). It is likely that the activation of the HPA

axis is one part of the integrated limbic response to complex information of a novel or threatening nature. Lesions of limbic structures such as the amygdala decrease ACTH or glucocorticoid responses to these more complex stressors but do not block the HPA responsiveness to systemic stressors such as ether induced respiratory distress which utilise direct catecholaminergic inputs to the PVN (Feldman *et al.* 1994, Herman & Cullinan 1997).

2.7.1.2.3 The amygdala

The amygdala, a limbic structure implicated in the regulation of HPA axis activity during stress, plays an important role in retrieval and emotional analysis of information pertinent to the stressor. If the stressor has an emotional component (i.e. psychological stressor) then the amygdala will stimulate the activity of the HPA axis and LC-NA/sympathetic system as well as prompting behavioural responses (Davis 1992). In rats, electrical stimulation of the amygdala elicits glucocorticoid secretion (Dunn & Whitener 1986), while lesion or ablation of the amygdala inhibits HPA responses to a variety of stressors including restraint, conditioned fear and acoustic or photic stimulation (Van de Kar *et al.* 1991, Feldman *et al.* 1994). The HPA axis can also influence the functioning of the amygdala. In contrast to the CRH containing neurons of the PVN, glucocorticoids function to increase CRH mRNA levels in the central nucleus of the amygdala (Watts 1996). This may function as a mechanism whereby activation of the stress response may increase fearfulness.

Though the amygdala plays a major role in the activation of the PVN during stress, anatomical studies show little evidence for direct neuronal projections between the two structures (e.g. rat, Prewitt & Herman 1998). It appears that at least in the rat, neurones from the amygdala do not project directly to the PVN but instead innervate PVN-projecting gamma-aminobutyric acid (GABA) producing cell groups in the bed nucleus of the stria terminalis (BNST), preoptic area and hypothalamus (Herman & Cullinan 1997). It is thought that amygdaloid projections which contain substantial populations of GABA and neuropeptide containing neurons, disinhibit PVN-projecting GABAergic neurons in the BNST,

preoptic area and hypothalamus, thereby, increasing the activity of the PVN resulting in HPA axis activation (Herman & Cullinan 1997).

2.7.1.2.4 The bed nucleus of the stria terminalis

The bed nucleus of the stria terminalis (BNST) is considered by many to be an extension of the central amygdaloid nucleus (Herman & Cullinan 1997). The BNST is the only limbic structure providing substantial direct innervation to the PVN. In lesioned rat studies, different areas of the BNST appear to have different roles in the control of the HPA axis. Subnuclei in the posterior region of the BNST convert excitatory signals from the hippocampus into a negative signal to the PVN, while subnuclei in the anterior BNST exert positive regulation on tonic HPA axis function (Herman *et al,* 1994).

2.7.1.2.5 The hippocampus

The hippocampus functions primarily as a region for associative learning and memory particularly in learning and memory tasks in which discontiguous items, in terms of their temporal and/or spatial positioning, must be associated (Wallenstein *et al.* 1998). Hippocampal influences upon the PVN are predominately inhibitory, mediated via GABAergic neurons that project to the BNST, preoptic area and hypothalamus. The hippocampus is believed to play a major role in the pathway by which glucocorticoids inhibit PVN activity. The hippocampus exhibits greater glucocorticoid binding, and levels of glucocorticoid receptors (both high affinity mineralcorticoid (MR) and low affinity glucocorticoid (GR) receptors) than any other brain structure (Herman 1993, Jacobson and Sapolsky, 1991).

The inhibitory role for the hippocampus in HPA regulation is supported by lesion studies in the rat and primate (Sapolsky *et al.,* 1984, Jacobson and Sapolsky, 1991, Sapolsky *et al.* 1991, Herman *et al.* 1995). These studies indicate that hippocampal lesion increases both the duration and magnitude of stress-induced glucocorticoid secretion while increasing the expression of CRH and AVP mRNA in parvocellular PVN neurons. Stimulation of the hippocampus also results in decreased HPA activity in both rat and human (Jacobson and Sapolsky, 1991).

Other structures in the limbic system, such as the prefrontal cortex or lateral septum also appear to inhibit the activity of the PVN. Lesion of these regions in the rat result in enhanced HPA responsiveness to acute stress such as restraint (Dobrakovova *et al.* 1982, Diorio *et al.* 1993).

2.7.1.3 Hypothalamic inputs

Inputs to the PVN also originate from other nuclei within the hypothalamus. These intrahypothalamic connections are generally inhibitory and act as substrates for integration of the HPA axis with other hypothalamic functions (i.e. circadian, autonomic, and behavioural regulatory mechanisms). Evidence for intrahypothalamic inhibition is provided from lesioning studies of rats where enhanced basal activity or HPA axis responses to stress (e.g. restraint) are observed following lesioning of selective hypothalamic nuclei, such as the arcuate nucleus, medial preoptic area, ventral nucleus or suprachiasmatic nucleus in the rat (Buijs *et al.* 1993, Larsen *et al.* 1994, Suemaru *et al.* 1995, Herman & Cullinan 1997).

An example of the important role hypothalamic inputs have in regulating the activity of the HPA axis is provided by the actions of the suprachiasmatic nucleus. Direct inputs from the hypothalamic suprachiasmatic nucleus to the PVN have been described (Berk & Finkelstein 1981) and are implicated in regulating the circadian rhythm in basal activity of the HPA axis. Lesions of the suprachiasmatic nucleus diminish (Abe *et al.* 1979), or abolish (Raisman & Brown-Grant 1977) the circadian rhythm in HPA axis activity in the rat.

2.7.1.4 Input from the lamina terminalis

Kovács & Sawchenko (1993) proposed a simple neural circuit by which blood-borne signals, such as osmotic information can alter gene expression in the magnocellular and parvocellular neurosecretory regions of the PVN. In the rat, chronic exposure to a hyperosmolar challenge (salt loading) induces differential alterations in CRH mRNA in the parvocellular (decreased expression) and magnocellular (increased expression) neurosecretory neurons. Pronounced induction of immediate-early gene product (Fos) also occurs in magnocellular

neurosecretory neurons and in several brain regions that have major projections to the PVN. These include a triad of cell groups making up the lamina terminalis of the third ventricle. Transections or lesions of the lamina terminalis projections to the PVN abolished the effects of salt loading in both the magno- and parvocellular regions of the PVN (Kovács & Sawchenko 1993).

2.7.1.5 Neurotransmitters involved in the regulation of the PVN

A number of different neurotransmitter systems have been implicated in the regulation of the PVN (reviewed in Whittnal 1992, Kovaks & Sawchenko 1996, Watts 1996, Herman & Cullinan 1997, Jessop 1999). These include the catecholamines noradrenaline and adrenaline which stimulate the release of CRH from the PVN (Liu *et al.* 1991). Plotsky (1987) demonstrated that noradrenaline produces a bell-shaped dose-response curve, with low doses stimulating CRH release via α_1-receptors and higher doses inhibiting CRH release via β-receptors. Acetylcholine and serotonin are also excitatory mediators participating in both the circadian rhythm and stress-induced release of CRH (Hillhouse & Milton 1988, Johnson *et al.* 1992). Acetylcholine stimulates CRH release via muscarinic cholinergic pathways as shown by the ability of arecoline (a muscarinergic agonist) to stimulate ACTH secretion in the rat (Calogero *et al.* 1989b). Serotonin (5-HT) enhances HPA axis secretion via 5-HT_{1A} and 5-HT_2 receptors (Calogero *et al.* 1989a).

The disinhibition of GABAergic neurons projecting to the PVN may play a critical role in stimulating the activity of the PVN during stress (Koeing 1999). The PVN is under tonic GABA inhibition from GABAergic neurons projecting from the BNST, the pre optic area and the dorsomedial nucleus. This inhibition is mediated through $GABA_A$ receptors (Keim & Shekhar 1996) and possibly $GABA_B$ receptors (Hausler *et al.* 1993). A role for GABA in regulating PVN activity during stress is supported by the finding that acute restraint and chronic stress increase the expression of the GABA-synthesising enzyme (glutamic acid decarboxylase) in specific regions of the brain associated with regulation of the PVN (e.g. BNST, hippocampus) (Bowers *et al.* 1998).

The pro-opiomelanocortin (POMC) derived peptides including the opioids (e.g. β-endorphin) and ACTH also plays an important role in modulating the activity of the PVN. Within the hypothalamus, POMC-containing neurons are primarily located in the arcuate nucleus. These neurons project to the PVN and also receive innervation from CRH containing neurones providing a closed feedback loop. During stress, activation of the arcuate nucleus by CRH release from the PVN results in the release of POMC peptides from axons projecting back to the PVN (Sawchenko *et al.* 1982, Nikolarakis *et al.* 1986, Burns *et al.* 1989). These POMC peptides limit the response of the HPA axis to stress by suppressing the synthesis of CRH in the PVN (Chrousos 1992, Chrousos & Gold 1992). The activation of POMC-containing neurons also plays an important role in stress induced analgesia (Chrousos 1995). Endogenous opiates have also been shown to attenuate the activity of the HPA axis in response to certain stressors (Rushen & Ladewig 1991) with β-endorphin and dynorphin exerting tonic inhibition of CRH release in the rat (Plotsky, 1986). Furthermore, administration of an opioid antagonist (naloxone) has been shown to increases the concentration of CRH and AVP in the pituitary venous blood of the horse (Alexander & Irvine 1995).

Other neurotransmitters and neuropeptides have also been implicated in stimulatory and inhibitory pathways controlling the activity of the PVN. Negative feedback effects of glucocorticoids, ACTH and CRH on CRH release have been demonstrated (Calogero *et al.* 1988). Other neuropeptides, such as leptin, substance P, somatostatin, oxytocin, gastrin, cholecystokinin, and pancreatic polypeptide, as well as bombesin, angiotensin II, galanin, neurotensin, enkephalins, neuropeptide Y, lipocortin-1, endothelin-1, natriuretic peptide, and from a common precursor, the vasoactive intestinal peptide (VIP) and the peptide histidine isoleucine (PHI) have also been implicated (Jessop 1999). The monoxide gases (nitric oxide and carbon monoxide) have also attracted considerable attention as potential modulators of HPA axis activity. It is thought that nitric oxide may exert a stimulatory effect on the synthesis of CRH within the PVN and an inhibitory effect upon its release from the median eminence depending upon whether the stressor is of a immune or nonimmune origin (Rivier 1998).

Several products of the immune system including Interleukin-l, Interleukin-2, Interleukin-6, or inflammatory mediators, such as platelet activating factor and tumour necrosis factor (TNF) also appear to stimulate CRH secretion from the PVN (Bernardini *et al.* 1989, Woloski *et al.* 1985, Johnson *et al.* 1992).

2.7.1.6 The response of the parvocellular PVN to stress

Within the parvocellular neurosecretory neurons of the PVN basal expression of the two main ACTH secretagogues (CRH and AVP) is low but can be induced or markedly upregulated by stress or adrenalectomy (Lightman & Young 1988). Acute stress induces a rapid release of CRH and VP into the pituitary portal circulation (rat, Plotsky 1991; horse, Alexander *et al.* 1996) as evidenced by the rapid depletion of CRH and AVP containing neurosecretory vesicles in the external lamina of the median eminence (Whitnall 1993). The stimuli that release CRH and AVP from the nerve terminals of the PVN parvocellular neurosecretory neurons also stimulate transcription of stress related genes in the nuclei of these neurons (Kovács & Sawchenko 1996). Apart from replenishing depleted neuropeptide stores, this mechanism may also serve to modulate responses to subsequent stressors (Kovács & Sawchenko 1996).

The PVN response to stress culminates in the release from axons projecting to the median eminence of a variety of ACTH secretagogues including CRH and AVP (Antoni, 1986; Keller-Wood and Dallman, 1984). In addition to CRH and AVP other factors including oxytocin, angiotensin II, cholecystokinin, vasoactive intestinal polypeptide, peptide histidine isoleucinamide, adrenaline and noradrenaline are released into the pituitary portal circulation to induce secretion of ACTH from anterior pituitary corticotrophs (reviewed in Antoni 1986).

2.7.1.6.1 Corticotropin-Releasing Hormone (CRH)

Corticotropin-releasing hormone (CRH) is a 41 amino acid peptide isolated and structurally characterised by Vale and co-workers (1981). The genes encoding the CRH molecule have been characterised in a number of species including humans (Shibihara *et al.* 1983), sheep (Vale *et al.* 1981, 1983) and rats (Rivier & Vale 1983) and appear to be highly conserved throughout evolution. The peptide

sequence of CRH also appears to be very homologous among species. For example, the CRH peptides from humans and rats are identical in amino acid sequence while human CRH differs from ovine CRH by only 7 amino acids (Vale *et al.* 1981, Rivier & Vale 1983, Shibihara *et al.* 1983) (Figure 2.7).

Rat/human	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII
Porcine	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMENF
Ovine	SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA
Bovine	SQEPPISLDLTFHLLREVLEMTKADQLAQQAHNNRKLLDIA

Figure 2.7 Comparison of the CRH(1-41) sequence from various species using the one letter code for amino acids (adapted from Fisher 1989).

CRH and CRH receptors are heterogeneously distributed throughout the CNS including the brainstem, midbrain, striatum, hippocampus, cerebral cortex and spinal cord, and are also present in sympathetic ganglia and the adrenal gland (De Souza *et al.* 1985, Suda *et al.* 1984). The broad distribution of CRH and its receptors in the CNS facilitates its role in co-ordinating the metabolic, cardiovascular and behavioural responses observed during stress. Intracerebral administration of CRH elicits complex behavioural and physiological responses including motor activation, increased grooming and decreased eating, increased arousal and anxiety and activation of the sympathetic branch of the ANS (Fisher 1989). These effects are not dependent upon activation of the HPA axis (Britton *et al.* 1986) and highlight the central actions of CRH. The highest density of CRH-containing neurones is found in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN) (Olschowka *et al.* 1982). These neurons function as the central focus for regulation of the HPA axis (Herman & Cullinan 1997).

The role of CRH in stimulating pro-opiomelanocortin (POMC) transcription, ACTH secretion, and mitosis in corticotroph cells of the anterior pituitary is well established in a number of species (Vale *et al.* 1983, Childs *et al.* 1995). The binding of CRH to CRH receptors in these cells activates adenylate cyclase increasing the intracellular levels of cAMP and enhancing the activity of cAMP

dependent protein kinases (Aguilera *et al.* 1983). This enzymatic cascade ends with the regulation of POMC gene expression and the release of POMC derived peptides such as ACTH, Lipotropin and β-endorphin into the circulation. Two different CRH receptors have been described in the rat and human, CRH_1 and CRH_2, which are both positively coupled to adenylate cyclase (Chalmers *et al.* 1996, Grigoriadis *et al.* 1996). CRH_1 receptors are found in high density in the pituitary, as well as the brain (particularly the neocortex). CRH_2 receptors of which two isoforms ($CRH_{2\alpha}$ and $CRH_{2\beta}$) have been identified in the rat and human, are more abundant in the periphery, but are also found in some brain areas such as the septum, ventromedial hypothalamus and dorsal raphe nucleus (Chalmers *et al.* 1996).

Some species (e.g. primates) also produce a specific plasma carrier protein for CRH termed CRH-binding protein. This protein was first identified in the circulation during late pregnancy in humans (Linton & Lowery 1986) and may function to limit the binding of CRH to its receptors and enhance the clearance of CRH from the circulation (Saphier *et al.* 1992). CRH-binding protein is also expressed in CRH target cells and may function to modulate the receptor mediated action of CRH (Behan *et al.* 1995).

Reduced pituitary responsiveness to CRH is characteristic of a number of human psychiatric disorders such as depression, panic disorder, anorexia nervosa and post-traumatic stress disorder (Smith & Hammond 1989). Chronic social stress in horses has been shown to reduce pituitary responsiveness (ACTH secretion) to an exogenous CRH challenge and to increase pituitary venous blood concentrations of CRH in animals at rest (Alexander *et al.* 1996). Responses to CRH challenge have not been reported for red deer, however, CRH is a potent stimulator of ACTH secretion by cultured pituitary corticotrophs from fallow deer (*Dama dama*) (Willard *et al.* 1995).

2.7.1.6.2 Arginine vasopressin (AVP)

Although AVP is primarily a product of the posterior pituitary gland it is also released from two sites in the hypothalamus: the parvocellular division of the paraventricular nucleus (PVN) where about 50% of the cells co-produce AVP

with CRH, and from the magnocellular neurons of the supraoptic nucleus (SON) and the PVN (Harbuz and Lightman, 1992, Scott & Dinan 1998).

The involvement of AVP in the control of ACTH secretion during various stress response paradigms is well established (Scott & Dinan 1998). The action of AVP on pituitary cells is mediated primarily by the V1 vasopressin receptor (the type present in the anterior pituitary is termed V1b) and to some extent by the V2 vasopressin receptor (reviewed in Kjaer 1993). Binding of AVP to its receptor activates phospholipase C increasing the intracellular levels of Ca^2+ and protein kinase C (Bilezikjian *et al.* 1987). Activation of cAMP by CRH is dependent upon protein kinases and may be a mechanism whereby AVP potentiates the activity of CRH (Bilezikjian *et al.* 1987).

It appears that the CRH component of the HPA axis is more sensitive to the suppressive effects of glucocorticoids than the AVP component (Petrides *et al.* 1997, Chrousos 1998). AVP plays an important role in maintaining corticotroph responsiveness in spite of high circulating levels of glucocorticoid during stress (Antoni 1993, Aguilera 1994). AVP appears to function in this manner by attenuating the inhibitory effect of glucocorticoids on CRH-stimulated ACTH secretion (Aguilera *et al.* 1994), and possibly by increasing the number of cells responsive to CRH, as shown in vitro (Childs and Unabia 1989). Chronic stress also increases the expression of AVP in the PVN (Antoni 1993). For example, approximately 50% of the medial-dorsal parvocellular CRH neurons responsible for ACTH secretion also express AVP in basal conditions, and this proportion increases markedly during adrenalectomy and chronic stress associated with hyper-responsiveness of the HPA axis (Figure 2.8) (Whitnall 1993). Chronic stress has also been reported to increase the pituitary binding of AVP (Aguilera *et al.* 1994). This would suggest that AVP may become the major regulator of ACTH release during chronic stress.

There is also increasing evidence that AVP may play a more dynamic role in the regulation ACTH secretion during basal and acute stress situations. In a number of species (e.g. horse, Irvine *et al.* 1989, Alexander *et al.* 1991), AVP secretion correlates more closely with ACTH levels than the secretion of CRH, suggesting a

more dynamic role for AVP in activity of the stress axis, and a primarily permissive function for CRH (Scott & Dinan 1998).

Interestingly, CRH and AVP appear not to affect the same pool of releasable ACTH in the anterior pituitary. Different populations of corticotrophs secrete ACTH in response to either CRH but not AVP, CRH or AVP, or CRH and AVP (Jia *et al.* 1991). Thus changes in the ratio of AVP to CRH secretion could affect HPA axis activity by altering the mix of corticotroph populations secreting ACTH (Whitnall 1992).

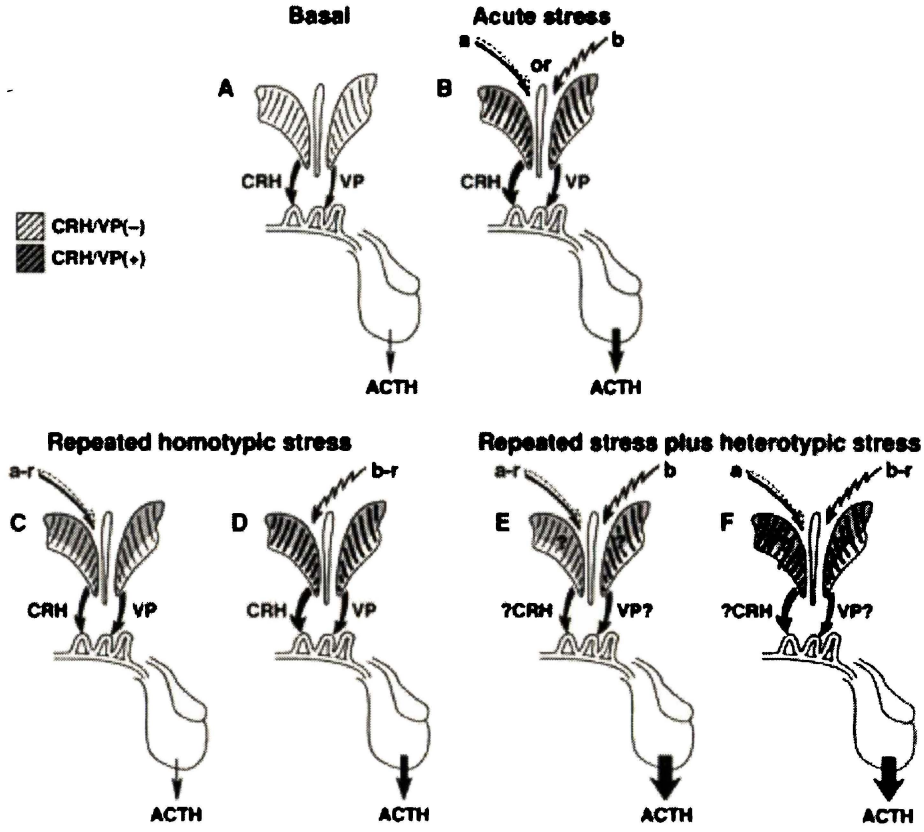


Figure 2.8 Schematic diagram of parvocellular neurons and pituitary corticotroph responses to acute stress (B), repeated stress (C and D) and repeated stress plus a superimposed acute heterotypical stress (E and F). Stress paradigms are classified as 'a' (immobilisation, cold) or 'b' (pain) according to their ability to desensitise corticotropin releasing hormone (CRH) and adrenocorticotropin (ACTH) during repeated stress (a-r and b-r). Under basal conditions (A), 50% of CRH neurons co-express vasopressin (VP; shaded area). The increases in CRH are shown by the thickness of the lines and the changes in VP expression by the size and intensity of the shaded area (stylised representation does not imply real distribution of VP-containing neurons). The question marks in (E) and (F) indicate that the effect of an acute heterotypical stress on CRH and VP expression and secretion in chronically stressed rats is unknown (Aguilera 1998).

2.7.2 The pituitary gland

The pituitary gland is located in a bony recess (sella turcica) at the Base of the brain. The pituitary gland has two distinct parts, the adenohypophysis (anterior pituitary) and the neurohypophysis (posterior pituitary) which are connected to the hypothalamus by the infundibulum "pituitary stalk" (Figure 2.9). Communication between the hypothalamus and pituitary include a neuroendocrine system that regulates the secretion of hormones from the anterior pituitary via the median eminence and portal vein system.

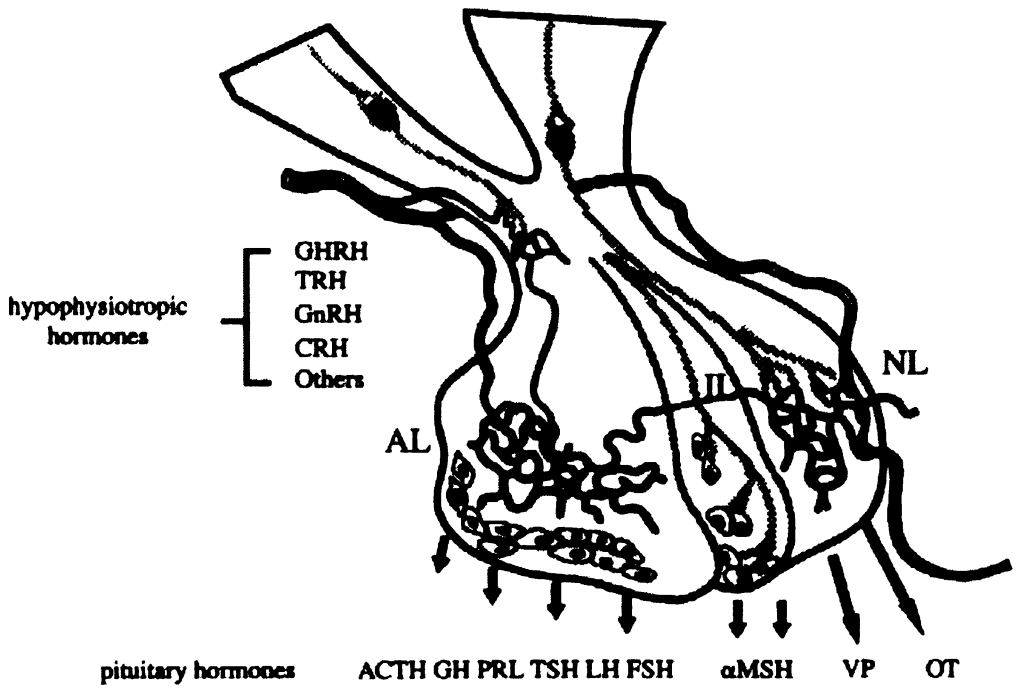


Figure 2.9 Schematic diagram of the relationship between the hypothalamus and the pituitary in a generalised mammal. The hypothalamus exerts control over the anterior lobe (AL) by releasing and inhibiting factors. These hormones reach the AL cells via capillaries of the pituitary portal system. The neural lobe (NL) of the pituitary is a downward projection of the hypothalamus. The intermediate lobe (IL) is under direct neurotransmitter control (Mol & Rijnberk 1997).

A neurosecretory pathway also exists between the anterior hypothalamus and the posterior pituitary. The posterior pituitary is actually an extension of the hypothalamus and contains axon terminals from hypothalamic neurons that synthesise and release oxytocin and vasopressin (Mol & Rijnberk 1997).

The anterior pituitary is derived from an evagination of region of oral ectoderm know as Rathke's pouch. In adults, the anterior pituitary is comprised of three distinct regions: the pars distalis, the pars intermedia which is poorly developed in higher mammals, and the pars tuberalis which constitutes a region wrapped around the infundibulum (Mol & Rijnberk 1997).

The major endocrine cell types of the anterior pituitary gland are the somatotropes which produce growth hormone (GH), the mammotropes which produce prolactin (PRL), thyrotropes which produce thyrotropin (TSH), the gonadotropes which produce luteinising hormone (LH) and follicle-stimulating hormone (FSH), and the corticotropes cells which produce pro-opiomelanocortin (POMC) derived peptides such as adrenocorticotropin (ACTH), β-endorphin (β-END), β-lipotropin (β-LPH) and α-melanotropin (MSH).

2.7.2.1 Pro-opiomelanocortin (POMC)

Pro-opiomelanocortin is the 35,000 dalton polyprotein precursor to a number of peptide hormones including ACTH, α-MSH, γ-MSH and β-endorphin (Figure 2.10). POMC is synthesised in the pituitary gland, brain (arcuate nucleus of the hypothalamus, zona incerta, lateral septum, nucleus accumbens, periventricular thalamus, periaqueductal gray, locus coeruleus, nucleus tractus solitarius, reticular formation, stria terminalis and medial amygdala), gastrointestinal tract and reproductive organs (Johnson *et al.* 1992). POMC gene expression in the corticotropes of the anterior pituitary is stimulated by the binding of corticotropin releasing factors such as CRH or AVP to specific membrane receptors which increase second messenger concentrations (the cAMP signal transduction system) within the cell. This results in changes in the phosphorylation of transcription factors which bind to a cAMP response element (CRE) in the promoter unit (a specific sequence of DNA on the 5' upstream part of the gene which permits the binding of transcription factors that enhance or inhibit gene expression). The synergistic stimulation of gene expression by CRH and AVP requires binding of yet partially characterised transcription factors in the distal and central part of the POMC promoter. POMC gene expression is inhibited by glucocorticoids binding to type II glucocorticoid receptors in the anterior pituitary corticotrope. A

glucocorticoid response element (GRE) in the promoter of the POMC gene binds the glucocorticoid receptor complex, resulting in inhibition of gene transcription.

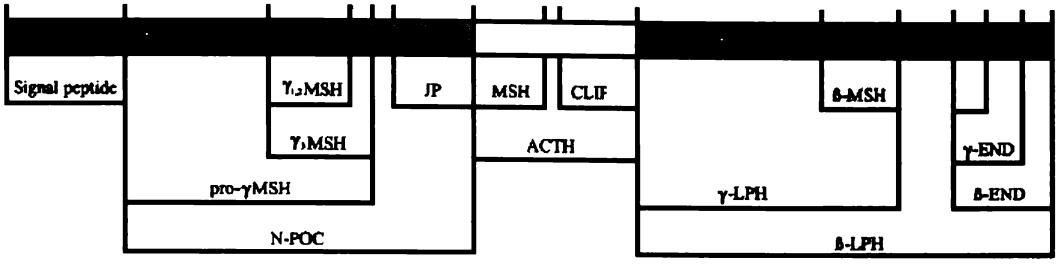


Figure 2.10 Schematic representation of preproopiomelanocortin (horizontal bar), with four main domains: the signal peptide, which is cleaved after entrance to the lumen of the rough endoplasmatic reticulum; the N-terminal peptide (N-POC), containing the (pro) γ-melanocyte stimulating hormone (γMSH) sequences and the joining peptide (JP); the ACTH domain, from which MSH and corticotropin-like intermediate lobe peptide (CLIP) can be generated; and the β-lipotropin (β-LPH) domain, including the endorphin (END) family of peptides and a metenkephalin sequence (Enk). Pairs of basic amino acid residues are indicated with vertical lines, representing potential sites of proteolytic cleavage. In the anterior lobe, major cleavage products are N-POC, ACTH, and β-LPH. In the intermediate lobe, the major products are N-POC, γ-MSH, α-MSH, and β-endorphin (Mol & Rijnberk 1997).

Following translation POMC is packaged and released into the cytoplasm as membrane bound granules. During storage of these granules in the cytoplasm, POMC undergoes post-translational modification involving specific proteolytic cleavage, C-terminal amidation, or N-terminal carboxylation. Post-translational modification of POMC varies depending on the species, the tissue, and the developmental stage of the animal and is determined by local proteolytic enzymes and pH. In the anterior pituitary POMC is processed to N-terminal (16K) peptide (N-POC), ACTH, and β-lipocortin (β-LPH) by the proteolytic enzyme PC1/PC3 (Mol & Rijnberk 1997). In the intermediate lobe these peptides undergo further processing with ACTH giving rise to α-MSH and corticotropin-like intermediate lobe peptide (CLIP), β-LPH giving rise to γ-LPH (containing β-MSH) and β-endorphin, and N-POC giving rise to a number of smaller peptides including γ-MSH (Keightley & Fuller 1996). The proteolytic enzyme PC2, present in both the anterior and intermediate lobes of the pituitary, generates the ACTH (1-13)

fragment, which after C-Terminal amidation and N-terminal acetylation forms α-MSH.

Differential processing of POMC also changes during foetal development. For example, the anterior pituitary of the human foetus produces mainly α-MSH and CLIP, but as term approaches ACTH production becomes predominant (Challis & Brooks 1989). The biologically active hormones are stored within the granules until the hormone is released by exocytosis involving the fusion of the granule membrane with the cell membrane. It is thought that peptides with such diverse biological activities might be contained within a single precursor to ensure the co-ordinated production and release of these hormones in response to stress (Keightley & Fuller 1996).

2.7.2.2 Adrenocorticotropic hormone (ACTH)

In the anterior pituitary POMC is broken down into ACTH, a 39 amino acid fragment (MW 4540). Sequence homology of the ACTH peptide is high between different species with the ACTH (1-24) sequence, necessary for full biological activity, identical among mammals (Figure 2.11). ACTH is released in an episodic fashion, as demonstrated in the pituitary venous effluent of the horse (Redekopp *et al.* 1986, Livesey *et al.* 1988, Alexander *et al.* 1994). In humans, pulses of ACTH are released an average of 40 times per 24 h period with a mean interburst interval of 39 min (Veldhuis *et al.* 1990). In the dog, episodic secretion of ACTH occurs an average of nine peaks per 24 h period (Kemppainen & Sartin 1984).

Human	SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEF	39
Rat	SYSMEHFRWGKPVGKKRRPVKVYPN**V**AE**N**ESAEAFPLEF	39
Mouse	SYSMEHFRWGKPVGKKRRPVKVYPN**V**AE**N**ESAEAFPLEF	39
Rabbit	SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLE**V**	39
Cattle	SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESA**Q**AFPLEF	39
Dog	SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFP**V**EF	39

Figure 2.11 Comparison of the ACTH(1-39) sequence from various species using the one letter code for amino acids (adapted from Mol & Rijnberk 1997).

The episodic secretion of ACTH reflects the pulastile release of CRH and AVP from the median eminence. Circadian rhythms in ACTH release have been described in a number of species including humans (Van Cauter *et al.* 1981, Veldhuis *et al.* 1990), pigs (Griffith & Minton 1991) and sheep (Guillaume *et al.* 1992b). In the human ACTH secretion exhibits a significant (3.8-fold) circadian rhythm, with maximum levels of ACTH in the morning (0818 h) (Veldhuis *et al.* 1990). However, this rhythm is evident only in the amplitude of ACTH pulses as their frequency remained consistent over the 24 h period (Veldhuis *et al.* 1990). Once released into the circulation, ACTH is rapidly metabolised. Estimates of the half-life of endogenous ACTH in the circulation vary between species (e.g humans 15 min, Veldhuis *et al.* 1990; rat 7 min, López & Negro-Vilar 1988).

2.7.2.2.1 ACTH action

ACTH is transported via the systemic circulation to the adrenal gland, where it stimulates steroidogenesis and secretion of glucocorticoids, aldosterone and adrenal androgens. The actions of ACTH at the adrenal cortex are mediated by binding to specific ACTH receptors. The adrenal ACTH receptor belongs to a family of five melanocortin-receptor subtypes that are positively coupled to G-protein and adenylate cyclase (Tatro 1996). The ACTH receptor (MC2-R) is expressed in the adrenal cortex and is selectively activated by ACTH (e.g. mouse, Schiöth *et al.* 1996; bovine, Liakos *et al.* 1998). Both glucocorticoids and ACTH have a stimulatory effect on the expression of ACTH receptors mRNA in the adrenal cortex (Penhoat *et al.* 1995, Picard-Hagen *et al.* 1997). ACTH also exerts a trophic (growth promoting) effect on the adrenal cortex, enhancing the response to subsequent stimulation. These mechanisms are believed to play a significant role in the increased ACTH receptor expression, adrenal hypertrophy and enhanced steroidogenic response to further hormonal stimulation seen during some situations of chronic stress (Johnson *et al.* 1992, Aguilera *et al.* 1996). Conversely, a reduction in ACTH secretion results in adrenal atrophy and an attenuated glucocorticoid response to ACTH, a condition also observed in some forms of chronic stress (Rhynes & Ewing 1973). Other, non-ACTH portions of POMC (the N-terminal POMC peptides) are also involved in adrenocortical growth (Estivariz *et al.* 1988).

ACTH also has effects other than stimulating the secretion of glucocorticoids. It has been reported to act in a short-loop negative feedback mechanism inhibiting CRH release (Calogero *et al.* 1988). ACTH is believed to also enhance attention, motivation, learning and memory retention (De Wied 1980). In pharmacological dosages, ACTH may also promote lipolysis in fat cells and amino acid uptake in muscle (Mol & Rijnberk 1997).

2.7.3 The adrenal gland

In mammals, the adrenal glands are bilateral structures located above the kidneys. At the centre of each gland, is the medulla (see section 2.6.1) which secretes adrenaline and noradrenaline. Surrounding the adrenal medulla is the adrenal cortex. This region can be divided histologically into three concentric zones (Figure 2.12). The outermost layer of the cortex is the zona glomerulosa consisting of small epithelioid cells arranged in ovoid groups immediately inside the capsule. These cells produce mineralocorticoids (aldosterone and deoxycorticosterone) that are critical for the maintenance of salt balance.

The zona fasciculata is the widest zone, accounting for 60 % of the cortex. Cells are cuboidal or columnar and form long cords, one or two cells thick, arranged radially to the organ capsule. These cells express the enzyme 17α-hydroxylase (P450c17) and synthesise glucocorticoids (cortisol and corticosterone). The innermost zone of the cortex is the zona reticularis, a continuation of the zona fasciculata, which comprises of a network of cell cords varying considerably in size and shape surrounding the large blood sinuses. These cells produce the adrenal androgens (androsenedione and dehydroepianodrosterone) as well as glucocorticoids and other hormones such as progesterone and estrogens.

The adult adrenal cortex undergoes permanent regeneration with adrenocortical cell division restricted to the outer quarter of the cortex (Zajicek et al. 1986, McNicol & Duffy 1987). The adrenocortical cells are progressively displaced centripetally until they reach the junction of the medulla (this process takes approximately 100 days in the rat (Zajicek et al. 1986)).

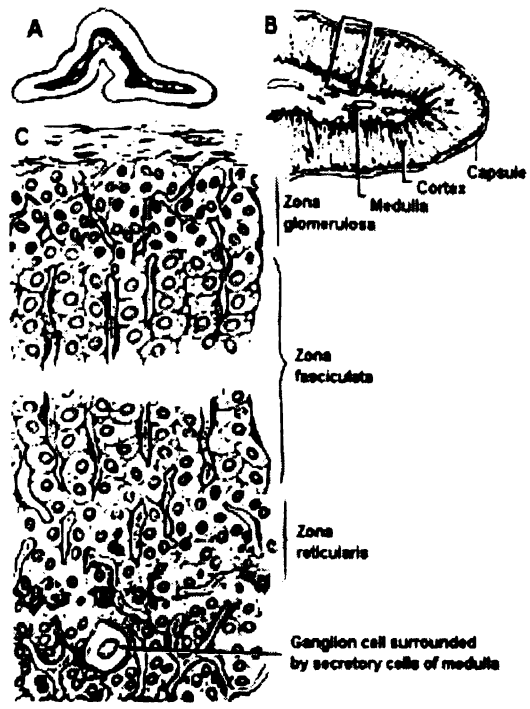


Figure 2.12 Diagrammatic representation of the adrenal gland. Cross section through entire adrenal gland (A), cross section under low magnification (B), and boxed area in B under higher magnification (C) (from Reece 1991).

Half the cells die on the way and the remainder are eliminated by apoptosis in the zona reticularis (Wyllie et al. 1973, Nussdorfer 1986). During their displacement, adrenocortical cells acquire the three distinct functional and morphological phenotypes characterised by different patterns of steroidogenic enzyme gene expression (Feige *et al.* 1998). Blood flow of the adrenal gland is also centripetal. This results in increasing steroid concentrations towards the centre of the gland which may contribute both to the changes in steroid production in the different zones, and to their eventual cell death (Hornsby 1982). High medullary glucocorticoid concentrations resulting from the centripetal blood flow also induce the enzymatic conversion of noradrenaline to adrenaline (Rijnberk & Mol 1997).

2.7.3.1 Steroidiogensis

The synthesis of adrenal steroids follows a pathway that begins with cholesterol. Some is synthesised from acetate, but most is taken up from low density

lipoproteins (LDL) (human) or high density lipoproteins (HDL) (rat) in the blood (Miller 1988). The biosynthesis of steroids begins with the enzymatic cleavage of a 6-carbon side chain group from cholesterol to form pregnenalone. This reaction is catalysed by the cytochrome P450 side chain cleavage enzyme ($P450_{scc}$), which is part of the cholesterol side chain cleavage enzyme system (CSCC) located on the matrix side of the inner mitochondria membrane in steroid producing cells (Stocco & Clark 1996). The adrenal CSCC is composed of a flavin adenine dinucleotide containing flavoprotein (adrenodoxin reductase) and a ferredoxin protein containing an iron sulfur cluster (adrenodoxin) which functions as a mini electron transport system to transfer reducing equivalents from NADPH to the $P450_{scc}$ (Miller 1988, Stocco & Clark 1996).

Once pregnenolone is formed, it may be metabolised within the mitochondria to progesterone by 3β hydroxysteroid dehydrogenase (3β-HSD) or it may exit the mitochondria and undergo further metabolism by microsomal steroid dehydrogenases and steroid P450 steroid hydroxylases (see Figure 2.13). The particular steroid hormone synthesised by a given cell type depends upon its complement of peptide hormone receptors, its response to peptide hormone stimulation and its genetically expressed complement of enzymes.

Although the pathway to pregnenolone synthesis is the same in all 3 zones of the cortex, the zones are histologically and enzymatically distinct, with the exact steroid hormone product depending on the enzymes present in the cells of each zone. The zona glomerulosa is the only region which contains the enzyme C-18 hydroxylase ($P450_{CMO}$ corticosterone methyloxidase) which converts corticosterone (a weak mineralocorticoid) to the principal and most potent mineralocorticoid, aldosterone. Glucocorticoids and adrenal androgens are exclusively synthesised in the cells of the zona fasciculata and zona reticularis. These cells contain the microsomal cytochrome $P450_{17\alpha}$ (17 α hydroxylase/lyase) which catalyses the 17α-hydroxylation of pregnenolone and progesterone as well as the side chain fission of 17α-hydroxy C_{21} steroids.

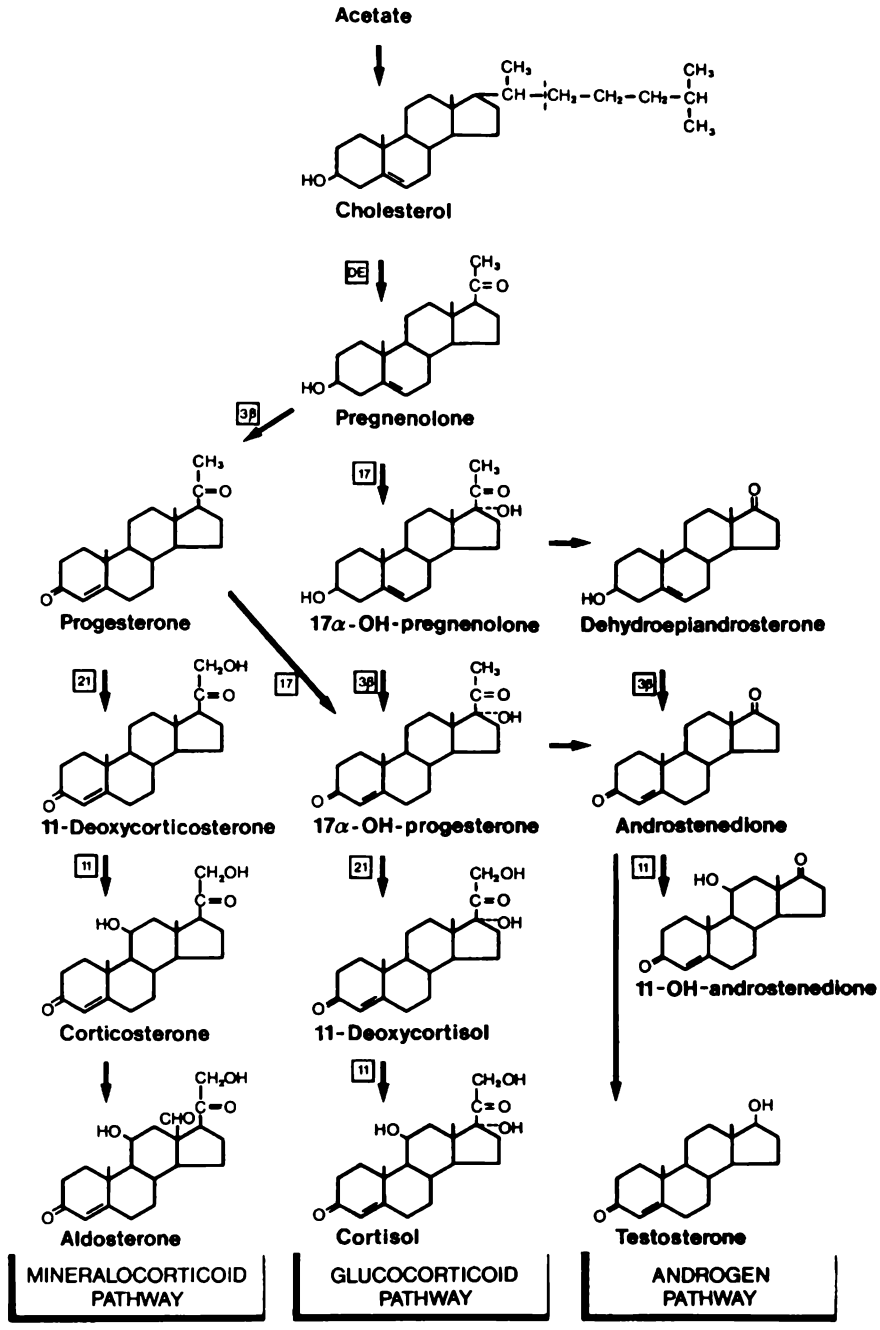


Figure 2.13 Biosynthetic pathways for adrenal steroid production. Letters and numbers by arrows denote specific enzymes: DE, debranching enzyme; 3β, 3β-ol-dehydrogenase with Δ4,5-isomerase; 11, C-11 hydroxylase; 17, C-17 hydroxylase; and 21, C-21 hydroxylase (Rijnberk & Mol 1997)

2.7.3.1.1 Steroid nomenclature

The steroids contain as their basic structure a cyclopentanoperhydro-phenanthrene nucleus consisting of three six-carbon rings (A, B and C) and a single 5 carbon ring (D) (Figure 2.14). Steroids include pro-vitamin D, cholesterol, bile salts,

cardiac glycosides as well as hormones of the adrenal cortex, the gonads and the placenta (Labhart 1986). Estrogens have 18 carbon atoms and are termed C_{18} steroids. They do not possess a side chain at C-17 and have a substituent at C-18.

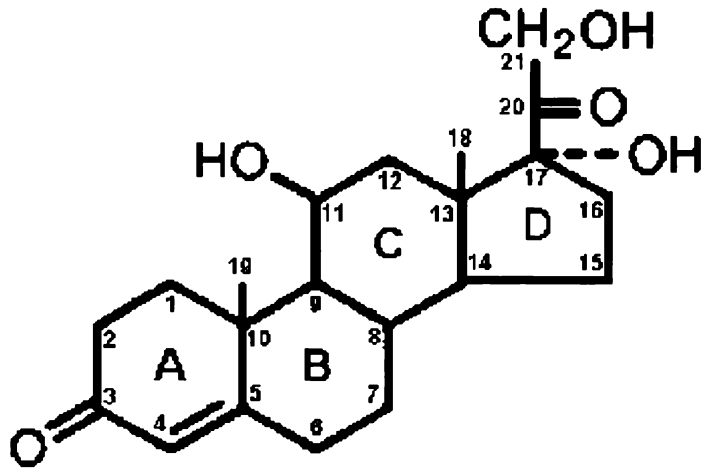


Figure 2.14 The numbers of the carbon atoms and the letters designating the rings of the cortisol molecule (IUPAC-IUB 1989).

The C_{19} steroids which have substituent methyl groups at positions C-18 and C-19 are androgens. The C_{21} steroids, the corticosteroids and progestagens have a two carbon side chains (C-20 and C-21) attached at C-17 and substituent methyl groups at C-18 and C-19. C_{21} steroids that also have a hydroxyl group at position 17 are termed 17-hydroxycorticosteroids and have predominately glucocorticoid properties (Rijnberk & Mol 1997).

2.7.3.2 Regulation of adrenal steroidogenesis

The anterior pituitary hormone adrenocorticotropin (ACTH) is the primary regulator of steroidogenesis in cells of the zona fasciculata, and zona reticularis of the adrenal cortex. Formation of pregnenolone is the first enzymatic step in steroidogenesis. However, the overall production of steroids is controlled by events that facilitate the transport of cholesterol from lipid droplets and other cellular stores to the mitochondrial outer membrane and its subsequent

translocation across the aqueous intermembrane space of the mitochondria to the inner membrane where the CSCC enzyme system is located (Stocco & Clark 1996). While both process are necessary to ensure maximal rates of steroidogenesis the key rate limiting step effected by ACTH stimulation is the delivery of the substrate, cholesterol, to the inner mitochondrial membrane (Stocco & Clark 1996, Nakamura et al. 1980).

Circulating ACTH stimulates steroid biosynthesis by binding to adenylate cyclase coupled ACTH receptors located in the plasma membrane of adrenocortical cells (Figure 2.15).. Activation of the adenylate cyclase enzyme results in the intracellular increase in cAMP and the activation of cAMP-dependent PKA. The increase in PKA results in the activation of two separate pathways. One pathway involves the activation of cholesterol ester hydrolase by phosphorylation. This enzyme converts cholesterol esters stored within lipid droplets in the cytoplasm to free cholesterol which is then available for translocation to the outer mitochondrial membrane, increasing the substrate available for steroidogenesis (Stocco & Clark 1996).

ACTH also regulates several proteins involved in cholesterol uptake by the cell, including cell surface lipoprotein receptors and 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is involved in cholesterol biosynthesis. ACTH also increases the intracellular transfer of free cholesterol to the outer mitochondrial membrane by a variety of mechanisms including altering the activity of regulatory proteins such as sterol carrier protein 2 (SCP_2) and morphological alterations in the architecture of the cell (Stocco & Clark 1996). In addition, ACTH is also able to increase steroidogenesis by stimulating the transcription of several key cAMP-responsive genes that encode steroidogenic enzymes (Miller 1988) and a regulatory protein involved in the transfer of the substrate cholesterol to the inner mitochondrial membrane and the site of CSCC enzyme system. This mitochondrial protein StAR (steroidogenic acute regulatory protein) is an essential component of the intramitochondrial cholesterol-transfer machinery and is believed to function as the key regulator of acute steroidogenesis (Stocco & Clark 1996).

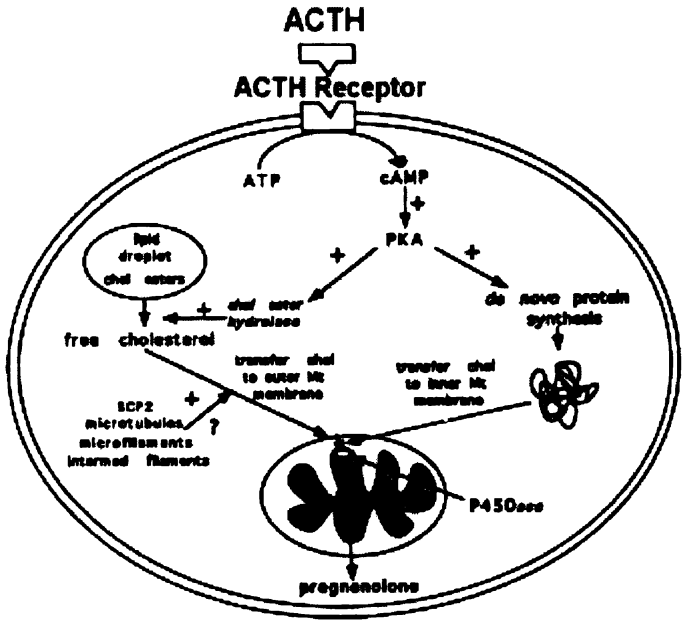


Figure 2.15 The effect of ACTH stimulation on adrenal steroidogenic cells (Stocco & Clark 1996).

ACTH has a trophic as well as stimulatory role in the regulation of the adrenal cortex. ACTH is the primary regulator of both fetal adrenal development and adult adrenal cortex homeostasis (Feige *et al.* 1998). Removal of ACTH trophic support by hypophysectomy or destruction of pituitary corticotrophs results in dramatic regression of fasciculata and reticularis zones of the adrenal cortex through apoptosis while the glomerulosa zone remains intact and functional in the rat (Carsia *et al.* 1996).

Adrenal function can be quantified *in vivo* by measuring the responsiveness of adrenal cortex, in terms of glucocorticoid output to challenge by exogenous ACTH. A number of studies have shown that conditions of chronic stress can alter the responsiveness of the hypothalamo-pituitary-adrenal (HPA) axis to ACTH challenge. For example, cattle exposed to crowding, social disruption, and competition for free stalls exhibit an increased cortisol response to exogenous ACTH (Friend *et al.* 1977, 1979). However, other studies have reported a reduced adrenal response to exogenous ACTH following chronic stress. For example,

cattle subjected to heat stress (Roman-Ponce et al. 1981) and tethering (Ladewig & Smidt 1989) respond to ACTH with a reduced cortisol increase.

The adrenocortical responsiveness to ACTH has been investigated in a number of deer species, including rusa (van Mourik & Stelmasiak 1984), white tailed (Smith & Bubenik 1990, Bubenik 1991), axis (Bubenik *et al.* 1991), fallow (Asher *et al.* 1989, Bubenik & Bartos 1993) and red (Goddard *et al.* 1994, Jopson *et al.* 1990, Bubenik & Bartos 1993, Hanlon *et al.* 1994, Suttie *et al.* 1995). In red deer, the adrenocortical response to an ACTH challenge has been reported to remain unchanged (Hanlon et al. 1994) or to decline (Goddard et al 1994, Pollard & Littlejohn 1998) during indoor housing, while chronic social stress in young hinds increases the cortisol response relative to baseline concentrations (Hanlon et al. 1995).

2.7.3.2.1 Intraadrenal regulation of steroidogenesis

A number of compounds other than ACTH can directly effect adrenal steroidogenesis (Figure 2.16). Intraadrenal concentrations of steroids may also influence steroidogenesis. Both $P450_{C21}$ and $P450_{C11}$ activity is affected by increased gonadal steroid concentrations with $P450_{C21}$ activity particularly suppressed by increased androgen concentrations (Hornsby 1982). Conventionally, the adrenal cortex and medulla have been viewed as distinct functional units, with cortical function regulated by ACTH and angiotensin II. However recent research has shown that the integrated control of adrenocortical function involves cortico-medullary interactions, the glands vascular supply, its neural input, the immune system, growth factors, and the intraglandular renin-angiotensin and CRH-ACTH systems (reviewed in Feige *et al.* 1998, Ehrhart-Bornstein *et al* 1998). These intraadrenal mechanisms play a role during development, differentiation, pathology and in the fine tuning of adrenocortical function.

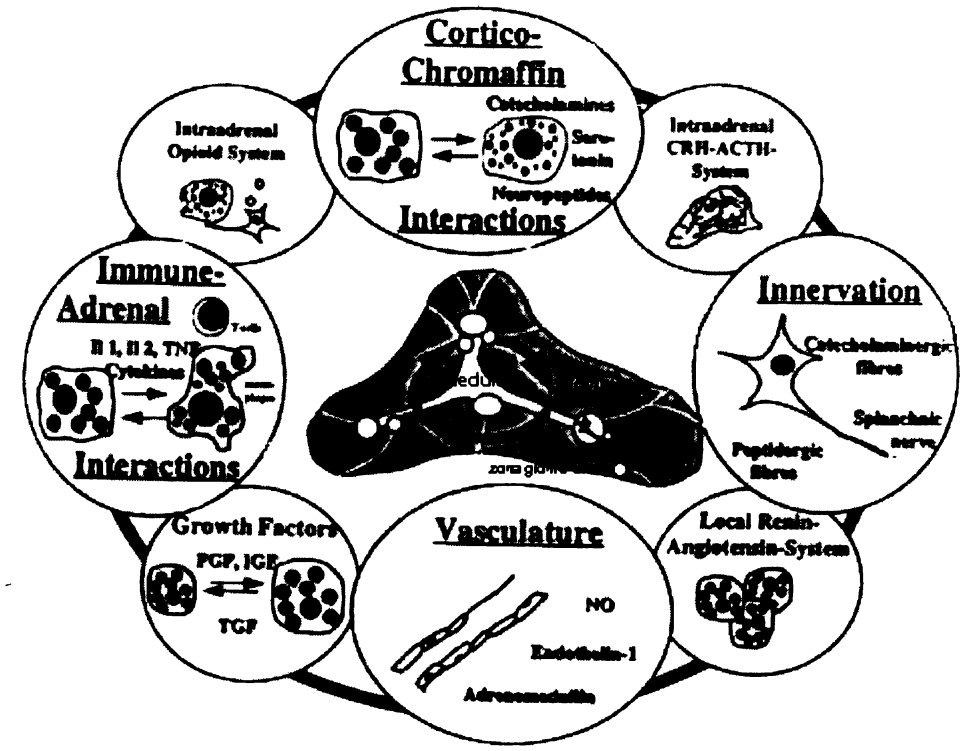


Figure 2.16 Intraadrenal regulation of adrenocortical steroidogenesis by the interaction of numerous cell types and systems (Ehrhart-Bornstein *et al.* 1998).

2.7.3.3 Bioavailability of secreted glucocorticoids

Many different steps are involved in the delivery of glucocorticoids to the glucocorticoid response elements within target tissues. The various stages are all points at which some modulation can occur in regulating the biological effects of glucocorticoids and hence the effects of stress.

2.7.3.3.1 Transport and plasma steroid binding

At normal concentrations, only about 10% of the total plasma cortisol and corticosterone are in the free biologically active form. Most of the glucocorticoids in circulation are bound to a glycoprotein (MW 52000) termed corticosteroid-binding globulin (CBG or transcortin) and to a lesser extent plasma albumin (Dallman *et al.* 1987b, De Kloet *et al.* 1998). CBG has a high affinity for glucocorticoids but a limited binding capacity while plasma albumin has low affinity and high capacity. Plasma binding to carrier proteins protects glucocorticoids from degradation by liver enzymes and filtration by the kidneys and provides a buffering system to prevent rapid changes in free cortisol

concentrations. Unbound glucocorticoids are considered biologically active, hence binding to CBG contributes to the regulation of the bioavailability of circulating glucocorticoids to target tissues (Coe *et al.* 1986).

Corticosteroid-binding globulin is also expressed intracellularly in the pituitary, liver, kidney, and lung (De Kloet *et al.* 1998). Within these organs intracellular CBG may bind intracellular glucocorticoids thus limiting their availability for glucocorticoid receptors. This is demonstrated in the pituitary where uptake of glucocorticoids in nuclei of corticotrophs is very low while the uptake of dexamethasone, which is does not bind to CBG, is high (De Kloet *et al.* 1975, 1977).

The binding affinities and points of saturation of CBG vary between species. In human plasma, 90% of plasma cortisol is bound to CBG whereas in domesticated species the proportion of cortisol bound to CBG ranges between 67 to 87 % depending upon the species (Gayrard *et al.* 1996). In humans, CBG is fully saturated at plasma cortisol concentrations of 200 ng/ml. Above this level, free cortisol increases to 20-30% of total cortisol and the remainder is bound to albumin (Labhart 1986).

Both acute (Fleshner *et al.* 1995) and chronic stress (Kattesh *et al.* 1980, Spencer *et al.* 1996, Alexander & Irvine 1998) can lower plasma cortisol binding capacity, though given the long circulatory half life of CBG (e.g. 14.5 h in rats, Smith & Hammond 1992) such changes are only apparent 24-48 h after stress (Dalin *et al.* 1993, Fleshner *et al.* 1995). The function of stress induced falls in binding is unclear but may serve to increase free glucocorticoid concentrations thereby increasing their effect on target tissues (Fleshner *et al* 1995). Thus, the interpretation of stress using only measurements of total cortisol concentrations in situations of acute and chronic stress must be treated with caution, as such results may not reflect the actual levels of glucocorticoids available to target tissues.

Glucocorticoid concentration may also play a role in regulating CBG concentrations as CBG concentrations increase following adrenalectomy and decrease following chronic high doses of exogenous glucocorticoids (Feldman *et*

al 1979, Smith & Hammond 1992). In addition, CBG synthesis is also regulated by immune activation associated with the acute phase response (Savu *et al.* 1980). The acute phase response collectively refers to the physiological changes that are initiated immediately following pathogen infection or tissue trauma which include a shift in liver metabolism from synthesis of carrier proteins (e.g. CBG) to production of acute phase proteins such as haptoglobin (Baumann & Gauldie 1994, Deak *et al* 1997). An acute phase response can also be initiated by stress and is thought to represent an anticipatory defensive immune response which may restrict infection, inflammation and injury produced by the threat (Deak *et al* 1997). Thus stress and elevated glucocorticoid concentrations can reduce CBG concentrations and hence the glucocorticoid binding capacity of plasma. Therefore, measurement of CBG concentrations or glucocorticoid binding capacity of plasma may offer another potential index of chronic stress.

There is also evidence that CBG, a member of the serine proteinase inhibitors (serpin) superfamily, promotes the targeted delivery of relatively high concentrations of free glucocorticoids to sites of inflammation (Pemberton et al. 1988, Hammond 1997). CBG is cleaved reducing its affinity to cortisol by a factor of 10 when exposed to serine proteases such as elastase on the surface of neutrophils (Rosner 1990). Once cleaved, CBG is removed from the circulation at a rate far exceeding the normal half-life (Rosner 1990).

The CBG-steroid complex may also act as a hormone, binding to high affinity specific CBG receptors present on the plasma membrane of a variety of cells (Rosner 1990). Once bound the CBG-steroid complex is internalised, adenylate cyclase is induced and intracellular cAMP accumulates (Rosner 1990, 1991). However, the biological consequences of CBG receptor activation have yet to be determined (Alexander & Irvine 1998).

2.7.3.3.2 Metabolic breakdown and excretion of glucocorticoids

Plasma glucocorticoid concentrations depend not only upon how much hormone is released from the adrenal cortex, but also upon their rate of removal from the blood. This removal, referred to as metabolic clearance, is due to uptake in target

organs, metabolism in the liver and excretion in the urine, milk, and saliva and via the gut (biliary). Cortisol is cleared from the plasma with a half-life of 60 min or less (Kraan *et al.* 1997). Only free cortisol is susceptible to inactivation by liver enzymes and ultrafiltration by the glomerulus of the kidneys (Ruckebusch *et al.* 1991). However only 20 % of the free cortisol filtered by the kidneys ends up being excreted in the urine as the rest is reabsorbed by the tubules (Ruckebusch *et al.* 1991). The kidney does however account for 50-80% of the excretion of metabolised steroids, the remainder being lost via the gut. The liver plays the primary role in the inactivation of steroid hormones and rendering them suitable for renal elimination.

In the hepatic microsomes glucocorticoids (cortisol and corticosterone) are reduced by $P450_{C11}$ to yield cortisone which is metabolically less active (Monder & White 1993). Further reduction of the double bond between C-4 and C-5 to form tetrahydro derivatives occurs and is probably the rate limiting step for glucocorticoid breakdown. These tetrahydro derivatives are first inactivated by conjugation with glucuronic acid to form water soluble glucucronidates. Depending on the species, conjugation to sulphates can also occur, as in the cat, which are mostly excreted via the biliary route (Rijnberk & Mol 1997).

2.7.4 Glucocorticoid receptors

The action of glucocorticoids are mediated by two types of receptors, the mineralocorticoid (MR or type I) receptor and the glucocorticoid (GR or type II) receptor (Rosenfeld *et al.* 1993). The MR is indistinguishable from the renal mineralocorticoid receptor in terms of *in vitro* binding specificity (cortisol ≈ corticosterone ≥ aldosterone >> dexamethasone). In rats, the equilibrium dissociation constant (K_d, the concentration of hormone that occupies half of the receptors) for corticosterone binding to MR is approximately 0.5 nM (Orchinik 1998). However, *in vivo* MR in tissues such as the kidney, liver, gastrointestinal tract, and salivary glands show a higher specificity for aldosterone than glucocorticoids due to the action of the enzyme 11-β-hydroxysteroid dehydrogenase (11βHSD). 11βHSD converts glucocorticoids to biologically inactive cortisone, thereby protecting the MR from being occupied by

glucocorticoids in mineralocorticoid target tissues such as the kidney, gastrointestinal tract, liver, spleen, skin and lungs (Wilckens 1995). Within the CNS aldosterone selective MRs are also expressed at hypothalamic sites involved in the regulation of salt appetite and autonomic outflow.

The majority of MR expression within the CNS is largely confined to areas outside of the hypothalamus including the neurons of limbic structures such as the hippocampus, septum, septohippocampal nucleus and amygdala as well as some brain stem nucleii. Importantly MRs within sites such as the hippocampus do not discriminate between corticosterone and aldosterone in terms of uptake and retention binding glucocorticoids with high affinity (approximately 10 fold higher than colocalised GRs).

Within the hippocampus a substantial proportion of high affinity MRs are occupied by glucocorticoids even during basal activity of the HPA axis. This would suggest that the tonic influences of glucocorticoids are exerted via occupancy of MRs functioning by proactive feedback on the HPA axis (De Kloet *et al.* 1998). This proactive feedback, resulting from glucocorticoids binding to MR, function to maintain basal activity of the HPA axis and control the sensitivity or threshold of the systems response to stress. Glucocorticoid binding to MRs promotes co-ordination of circadian events, such as the sleep/wake cycle and food intake and is involved in processes underlying selective attention, integration of sensory information, and response selection (De Kloet *et al.* 1998).

The GR is the prototypical receptor for glucocorticoids in the liver and exhibits a higher binding specificity for glucocorticoids than mineralocorticoids (dexamethasone > cortisol > corticosterone >> aldosterone). In the rat, GR has approximately 10-fold lower affinity for corticosterone than does MR (K_d approximately 5 nM) (Orchinik 1998). The GR receptors is widely distributed throughout the CNS and can be found in particularly high concentrations within limbic structures associated with the organisation of the stress response such as the PVN and hippocampus. The main function of GR is the curtailment of the stress-induced HPA axis hyperactivity through inhibition of CRH, AVP, cytokine

and POMC synthesis and facilitation of information storage, fear and food motivated behaviours (De Kloet 1991).

The difference in affinity between MR and GR for glucocorticoids has led to the concept that these receptors constitute a two-tiered system in which MR is optimised for responding to basal levels of glucocorticoids to maintain HPA axis activity, while GR is optimised for responding to the circadian peak and stress induced levels of glucocorticoids and is primarily involved in to mediate (Dallman 1993, Orchinik 1998). There is also reported to be significant interactions between the two-receptor systems (reviewed in de Kloet *et al.* 1998). However, this two-tiered model has recently been criticised on the basis of receptor occupancy (for details see Orchinik 1998). Questions on how the GR system can differentiate the high stress induced levels of glucocorticoids when 90% of the GR is occupied during the circadian peak in rats have yet to be answered (Orchinik 1998).

A receptor with lower affinity for glucocorticoids than GR may also exist. A binding site in rat synaptic membranes with a Kd near 100 nM for corticosterone has been described (Sze & Towle 1993). Such a membrane bound neuronal receptor could provide a mechanism for rapid responses to acute fluctuations in glucocorticoid levels as well as being far better suited to distinguishing between corticosterone levels produced during daily peaks or mild and severe stressors (Orchinik 1998).

The glucocorticoid receptor regulates gene expression by at least two mechanisms. By their lipophilic nature, glucocorticoids can easily cross the cell membrane. Binding of the hormone to GR induces a conformational change of the receptor leading to its release from a complex with heat-shock proteins (e.g. hsp90 and hsp56) and translocation into the nucleus. Heat shock proteins by complexing with the free glucocorticoid receptor influence glucocorticoid efficacy by interfering with the binding of glucocorticoids to these receptors and regulating the trafficking of receptors between the cytoplasm and the nucleus (Kang et al. 1994, Wilckens 1995). Within the nucleus the receptor either binds as a dimer to glucocorticoid response elements and activates transcription or interferes as a

monomer with transcriptional gene activation by other factors (e.g. AP-1 and nuclear factor $_{\kappa}$B) (Figure 2.17) (Tronche *et al.* 1998). Studies have also shown competition between steroid receptors for nuclear factors involved in gene regulation (e.g. glucocorticoid type II receptor and the estrogen receptor competing for the nuclear factor $_{\kappa}$B) (Meyer et al. 1989, Ray & Prefontaine 1994).

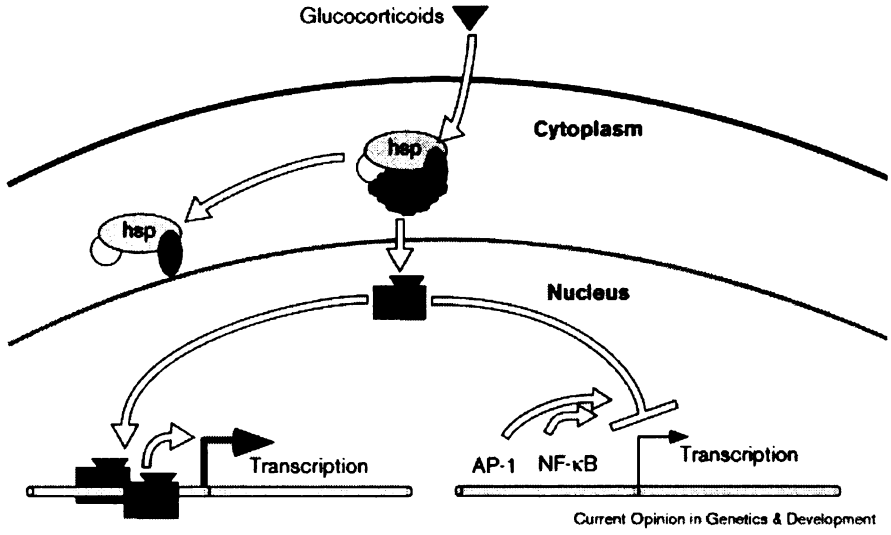


Figure 2.17 Molecular mechanisms of glucocorticoid action on gene expression. Following translocation to the nucleus the receptor-ligand complex either binds as a dimer to glucocorticoid response elements and activates transcription or interferes as a monomer with transcriptional gene activation by other factors (e.g. AP-1 and nuclear factor $_{\kappa}$B) (Tronche *et al.* 1998).

2.7.5 Function of glucocorticoids

Glucocorticoids have a wide range of actions including effects on metabolism, cardiovascular and muscle function, growth, immune function, reproductive function and behaviour. Their effects are so crucial for maintaining metabolic homeostasis that death will occur following adrenalectomy (removal of the adrenal glands) unless exogenous glucocorticoids are administered or the animal is kept under stress free conditions (Hayes & Murad 1992).

Glucocorticoid act on target cells by diffusing through the cell membrane, binding to the cytoplasmic GR or MR glucocorticoid receptors, translocating to the nucleus and the subsequent activation or inhibition of specific genes. The actions

of glucocorticoids can be grouped into permissive and regulatory effects (Ingle 1954). The permissive role of glucocorticoids is crucial for maintenance of homeostasis during basal states (Munck & Guyre 1986). They permit other hormones and factors to accomplish their function at a normal level by acting as 'biological amplifiers' of metabolic processes (Granner 1979). For example, the ability of catecholamines to increase the rate of lipolysis in adipose tissue requires the presence of glucocorticoids (Hayes & Murad 1992).

The regulatory effects of glucocorticoids are exerted only by stress induced levels and function to restrain defence reactions to stress, preventing overreaction of the central stress, immune and other systems which would become damaging if left unchecked (see Munck *et al.* 1984 for review). For example, glucocorticoids possess an antiinflammatory effect, inhibiting the production or action of a number of cytokines and other mediators of inflammation. Thus a reduction in the glucocorticoid response to stress may result in increased susceptibility to autoimmune or inflammatory diseases (Chrousos 1995).

2.7.5.1 Metabolic actions of glucocorticoids

Although glucocorticoids act upon many tissues, one of the primary functions of glucocorticoids is in the regulation of cells involved in metabolism (e.g. liver hepatocytes). The actions of glucocorticoids are catabolic, causing mobilization of energy stores and stimulating the synthesise of glucose and glycogen from non-carbohydrate precursors in a process termed gluconeogensis (Exton 1979). Glucocorticoids stimulate liver hepatocyte cells to produce gluconeogenic enzymes such as fructose-1-6-diphosphatase and glucose-6-phosphatae that enhance the conversion of protein to glucose (Ruckebusch *et al.* 1991). Glucocorticoids also increase the availability of gluconeogenic substrates by increasing the net release of amino acids from peripheral tissues and elevating plasma free fatty acids concentrations by stimulating lipolysis in adipocytes (Dunn & Kramary 1984). In addition to enhancing glucose and glycogen production by the liver, glucocorticoids increase energy availability by inhibiting glucose uptake by cells not essential to the stress response (De Kloet & Veldhuis 1985, Sapolsky 1994).

Chronic elevations in glucocorticoids (primary hypercortisolism or Cushing's syndrome) are associated with polyphagia, obesity, fatigue, muscle wasting, insulin resistance, hypertension and elevated cholesterol and triglyceride levels (Stratakis *et al.* 1995, Peeke & Chrousos 1995). Some of these anabolic effects are due in part to the stimulatory effect glucocorticoids have on insulin secretion. Insulin has reciprocal effects to that of glucocorticoids on energy storage (anabolic verses catabolic effects, respectively) and feeding (insulin inhibits and glucocorticoids stimulate feeding) (Dallman *et al.* 1993, Dallman *et al.* 1995). Under normal conditions these two hormones regulate overall energy balance. However in hypercortisolism, stimulation of insulin secretion by glucocorticoids favours fat synthesis and deposition.

Glucocorticoids also exert multiple suppressing effects on somatic growth (Dieguez *et al.* 1996). Glucocorticoids play an important role in the regulation of growth hormone (GH) synthesis and secretion with reduced GH responses to stimulation tests in conditions of chronic exposure to excessive cortisol secretion (Dieguez *et al.* 1996). They also exert effects upon metabolic processes essential for normal growth (e.g., bone formation, nitrogen retention, collagen formation) (Allen 1996, Ziegler & Kasperk 1998). For example, glucocorticoids effect bone formation by diminishing calcium absorption and increasing renal calcium excretion. This results in a negative calcium balance, secondary hyperparathyroidism, osteoclast activation and impairment of osteoblast activity which further contributes to bone loss (Ziegler & Kasperk 1998).

2.7.5.2 Glucocorticoids, stress and immune function

The links between stress, reduced immune function and increased susceptibility to disease has been demonstrated in a variety of species including humans (Lee *et al.* 1995), sheep (Minton *et al.* 1992), cattle (Mackenzie *et al.* 1997), horses (Hines *et al.* 1996), poultry (Dietert *et al.* 1994), mice (Padgett *et al.* 1998) and fish (Maule *et al.* 1989). This subject has also been extensively reviewed in the literature (e.g. Rabin *et al.* 1989, Minton 1994, Griffin & Thomson 1998, Rozlog *et al.* 1999).

In red deer, only a handful of studies have investigated the effects of stress on immune function and disease resistance. These studies have shown that stressors such as capture from the wild (Griffin *et al.* 1991), transport and relocation (Thomson *et al.* 1994) and social stress induced by repeated changes in herd composition (Hanlon *et al.* 1995) suppress immune function. In addition weaning of red deer fawns has been shown to either enhance (Hibma & Griffin 1994) or suppress (Griffin *et al.* 1991) aspects of immune function (e.g. antibody formation).

Although stress-induced immunosuppression is likely to be mediated through many different factors (Khansari *et al.* 1990, Minton 1994, Stanisz 1994) and hormones (Gala 1991, Stanisz 1994), glucocorticoids are generally considered to be one of the most important (Munck *et al.* 1984) as they regulate virtually every component of the immune and inflammatory response (Griffin & Thomson 1998). Glucocorticoid actions include influences on cytokine production, total numbers of lymphocytes and their subpopulations in the circulation, the acute phase response, thymocyte growth and differentiation, and restraining the immune/inflammatory response (reviewed in Munck *et al.* 1984, Munck & Guyre 1991, Chrousos 1995, Wilckens & De Rijk 1997, Griffin and Thomson 1998).

One of the most potent anti-inflammatory actions of glucocorticoids is the suppression of cytokine production (Munck & Náray-Fejes-Tóth 1994, Auphan *et al.* 1995, Chrousos 1995). For example, the release of proinflammatory cytokines interleukin-1, interleukin-6, interleukin-8 and tumour necrosis factor α from macrophages is suppressed by glucocorticoids (Zwilling 1994). By suppressing the production and release of cytokines, glucocorticoids can suppress the growth and differentiation of immune cells, along with the induction of their specific functions, such as migration, proliferation and the activation of effector cells such as macrophages, natural killer cells, and cytotoxic T cells (Griffin & Thomson 1998).

However, it should be noted that many of these inhibitory effects are observed following supraphysiological doses of glucocorticoids (Wilckens 1995). There is increasing evidence that physiological concentrations of glucocorticoids may be

necessary for the development and maintenance of normal immunity (reviewed by Jefferies 1991).

Glucocorticoid receptors are found in the cytoplasm of all the major subsets of lymphocytes including granulocytes, monocytes/macrophages and both T-cell and B-cell lymphocytes (Griffin & Thomson 1998). Administration of glucocorticoids causes a reduction in the number of lymphocytes, monocytes, eosinophils, and basophils in the peripheral circulation while neutrophils are increased (neutrophilia). These changes peak within 4-6 h and reflect, depending upon the species, either cell death of lymphocytes or a redistribution of leukocytes between the blood and other immune compartments such as the skin, lymph nodes and bone marrow (Dhabhar *et al.* 1995, 1996, Dhabhar & McEwen 1996, 1999, Griffin & Thomson 1998). In species where redistribution occurs (e.g. rat), trafficking of leukocytes to the skin has also been associated with immunoenhancement of delayed-type hypersensitivity reactions in acute stress (Dhabhar *et al.*1996, Dhabhar & McEwen 1996, 1999). These reactions are antigen-specific cell-mediated immune responses that, depending on the antigen, mediate beneficial (e.g. resistance to viruses, bacteria, and fungi) or harmful (e.g. allergic dermatitis and autoimmunity) aspects of immune function. In the rat, corticosterone, acting at the type II glucocorticoid receptor, is a major mediator of the stress-induced changes in blood lymphocyte and monocyte distribution (Dhabhar *et al.* 1996). Thus glucocorticoids released during an acute stress response may help prepare the immune system for potential challenges in the periphery (e.g., wounding or infection) for which stress perception by the brain may serve as an early warning signal (Dhabhar & McEwen 1999). Therefore endogenous glucocorticoids appear not only to suppress but also direct and enhance immune functions depending on their concentration. This may be achieved by acting via distinct mineralocorticoid receptor and glucocorticoid receptor pathways (Wiegers *et al.* 1993, Wilckens & De Rijk 1997).

The communication between the immune system and HPA axis is bi-directional. Immune system activation during infectious disease, autoimmune inflammatory disease or trauma stimulates HPA axis activity. Communication is achieved via the actions of the proinflammatory cytokines (tumour necrosis factor-α,

interleukin-1, and interleukin-6) that either act individually or in synergy to stimulate the HPA axis (Perlstein *et al.* 1993). This is mediated through stimulation of hypothalamic CRH and AVP secretion and by direct effects on the pituitary corticotroph and adrenal cortex (Stratakis *et al.* 1995). Two way communication between the HPA axis and immune system is essential for keeping both systems in balance. Disruption of this balance can lead to the development of disease. For example, an excessive HPA axis response to inflammatory stimuli or hypercortisolism will suppress immune function resulting in an increased susceptibility to infection. Whereas, an inadequate HPA axis response to inflammatory stimuli will produce an overactive immune response increasing the susceptibility to autoimmune inflammatory disease (Cizza & Sternberg 1994). Evidence that this bidirectional communication plays a vital role in susceptibility to inflammatory and infectious disease is derived largely from animal models such as the Fischer and Lewis rats, selected for their resistance or susceptibility to inflammatory disease. Sternberg *et al.* (1992) demonstrated that susceptibility to streptococcal cell wall induced arthritis in Lewis rats is related to a lack of glucocorticoid restraint of inflammation. Whereas, Fischer rats exhibit greater glucocorticoid responses to inflammation and are relatively resistant to streptococcal cell wall induced arthritis. The difference between the two strains is due to proinflammatory cytokines being less effective in stimulating CRH synthesis and secretion in the Lewis rat (Sternberg *et al.* 1992).

2.7.5.3 Glucocorticoids and reproductive function

Various components of the HPA axis inhibit reproductive function at all levels of the hypothalamic-pituitary-gonadal axis (Rabin *et al.* 1988, Magiakou *et al.* 1997). For example, CRH inhibits the secretion of gonadotrophin hormone-releasing hormone (GnRH) by the arcuate neurons of the hypothalamus, either directly or via the stimulation of POMC secreting neurons of the arcuate nucleus (Stratakis *et al.* 1995). Glucocorticoids also exert inhibitory effects at the level of the GnRH neurons, pituitary gonadotrophs and the gonads themselves and render target tissues of sex steroids resistant to these hormones (Rabin *et al.* 1988, Chrousos & Gold 1992).

2.7.5.4 Behavioural effects of glucocorticoids

Glucocorticoids alter brain function causing behavioural changes during exposure to a stressor. In the short term glucocorticoids have been reported to cause arousal and euphoria in humans, possibly by stimulating CRH production in the central nucleus of the amygdala and the production of dopamine by neurones of the mesocorticolimbic system (Chrousos 1995). High levels of glucocorticoids are also associated with elevated indices of fear in the rat (Takahashi & Kim 1994, Schulkin *et al.* 1998). Glucocorticoids increase CRH gene expression in the central nucleus of the amygdala, a site linked to fear and anxiety (Davis *et al.* 1997, Schulkin *et al.* 1998).

Glucocorticoids also appear to facilitate behavioural transitions, producing a shift in the dominant sensory modality by rapidly modifying the processing of sensory information (Orchinik 1998). Glucocorticoids facilitate redirection of behaviour towards behaviours for dealing with threatening external stimuli such as escape, aggression or vigilance (Orchinik 1998). These changes are dependent upon the neuroendocrine context such as the pattern of circulating glucocorticoids or the neurochemical systems activated by the stressors (Orchinik 1998). Over a longer period, glucocorticoids cause the atypical depression seen in Cushing's syndrome, presumably by suppressing hypothalamic CRH secretion (Chrousos & Gold 1992, Chrousos 1995).

Glucocorticoids also play a role in influencing cognitive function (reviewed in Lupien & McEwen 1997). Previous studies have shown that stress and glucocorticoids influence acquisition and long term storage of newly acquired information (Kirschbaum *et al.* 1996, Luine *et al.* 1996) as well as memory retrieval (Quervain *et al.* 1998). The effects of stress and glucocorticoids on memory consolidation follow an inverted-U-shape dose-response relationship. Extreme high and low levels may impair consolidation, while intermediate doses enhance memory (Lupien & McEwen 1997). It also appears that activation of glucocorticoid receptors may be a prerequisite for the long-term storage of information (Quervain *et al.* 1998). Once memories are consolidated,

glucocorticoid levels at the time of recall may also effect the efficacy or accuracy of the information retrieved (Quervain *et al.* 1998).

2.7.5.5 Glucocorticoid negative feedback regulation of the HPA axis

A variety of different mechanisms are involved in restraining the activity of the HPA axis. These include inhibitory neural modulation as well as various neuroendocrine negative feedback loops (Keller-Wood & Dallman 1984, Calogero *et al.* 1988). The neural pathways, neurotransmitters, neuropeptides and second messengers involved in the neural regulation of HPA axis activity have been discussed in previous sections and are reviewed in Kovaks & Sawchenko (1996), Watts (1996) and Herman & Cullinan (1997).

The main feedback regulation of the HPA axis involves the negative feedback control loops mediated by glucocorticoids (Figure 2.18). The function of glucocorticoids and their receptors in mediating an inhibitory feedback signal to the hippocampus, hypothalamus and pituitary to restrain central drive to the HPA axis has been extensively reviewed (e.g. Keller-Wood & Dallman 1984, De Kloet *et al.* 1998). The importance of glucocorticoid mediated feedback in regulating HPA axis activity is evidenced by the effect adrenalectomy has on HPA axis activity. Adrenalectomy results in the removal of endogenous glucocorticoid feedback and produces hypersecretion of ACTH and a corresponding increase in POMC mRNA expression in the anterior pituitary of the rat (Birnberg *et al.* 1983). Secretion of CRH and AVP from the median eminence into the portal vein system is also increased (Fink *et al.* 1988), as is the production of these peptides in the PVN (Kovács *et al.* 1986). In addition to increased basal secretion of ACTH and its secretagogues, the HPA axis exhibits increased amplitude in circadian changes and greater responses to stress following adrenalectomy (Dallman *et al.* 1987a). These changes are reversed or prevented by the administration of exogenous glucocorticoids (Harbuz & Lightman 1992).

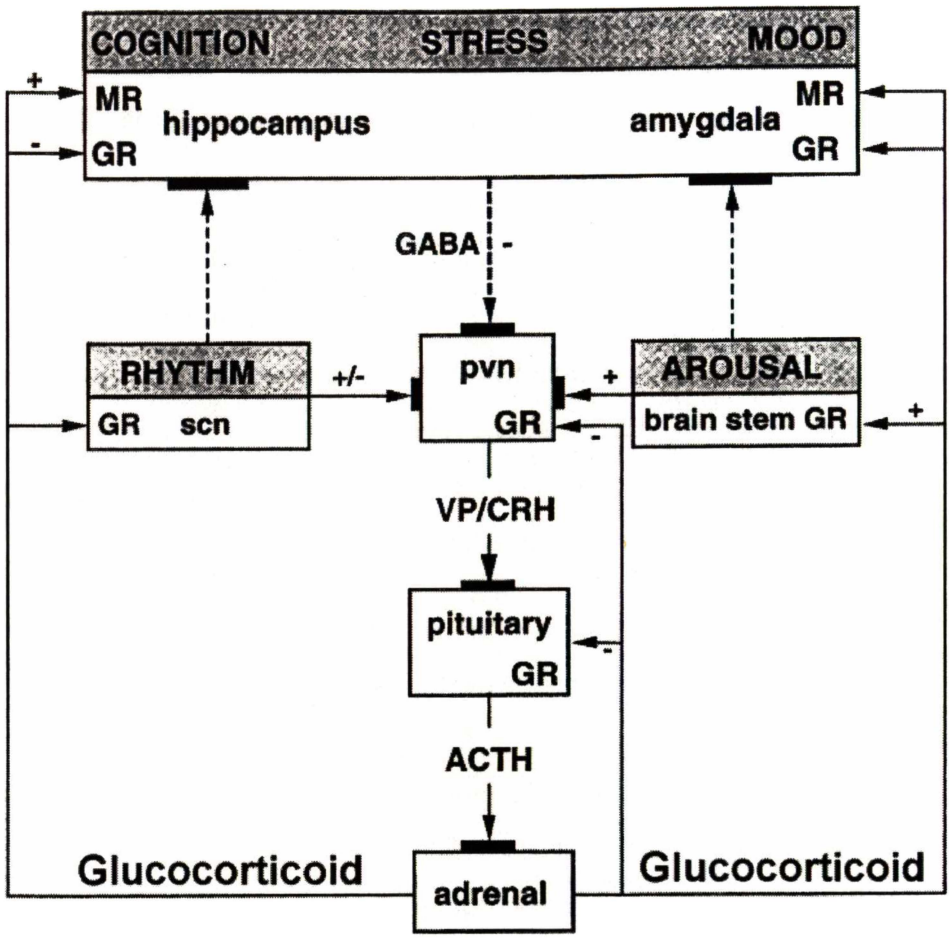


Figure 2.18 Glucocorticoid feedback regulation of the HPA axis. The diagram shows the organisation of the HPA axis and in simplified form, some of its major neuronal afferents. Neural activation of PVN neurons in response to visceral stimuli occurs via monosynaptic catecholaminergic projections arising from brain stem nuclei. Projections of the suprachiasmatic nucleus transfer excitatory as well as inhibitory information to ensure circadian rhythmicity of the HPA axis. In hypothalamus, preoptic area, and bed nucleus of the stria terminalis (BNST) a γ-aminobutyric acid (GABA) network conveys an inhibitory input to the PVN neurons. This inhibitory GABA-ergic tone is enhanced by excitatory hippocampal output. GABA-ergic inhibitory tone is inhibited (and thus becomes excitatory) through a GABA input from the central amygdala. MRs mediate potentiation of hippocampal output, and thus enhance neural inhibition of HPA axis, while via GRs glucocorticoids dampen hippocampal output, which thus leads to HPA disinhibition. GRs also mediate the activation of excitatory input (NE, E, 5HT) to PVN from brain stem. GRs in PVN and pituitary mediate the blockade of stress-induced HPA activation (De Kloet *et al.* 1998).

Glucocorticoids inhibit activity of the HPA axis by feeding back to various levels of the axis, including suprahypothalamic levels, such as the hippocampus, as well as in the PVN, median eminence and the pituitary corticotroph (Spinedi *et al.* 1991, reviewed in Watts 1996). . In addition to these more central sites, extremely high levels of glucocorticoids are also thought to have a direct inhibitory effect on adrenal steroidogenesis (Labhart 1986).

There is strong evidence that glucocorticoids exert part of their negative feedback effects at the level of the PVN. Administration of glucocorticoids into the PVN region down-regulates CRH mRNA and decreases ACTH secretion (Whitnall 1993). Similar reductions in CRH mRNA levels are seen when dexamethasone is administered either systemically or locally into the PVN (Kovács *et al.* 1986, Kovács & Mezey 1987, Harbuz & Lightman 1989). The capacity for direct feedback at the level of the PVN is supported by evidence for expression of GR receptors in CRH containing neurones of the PVN (Uht *et al.* 1988). Other hypothalamic sites also express GR receptor protein and mRNA and may also function as sites for glucocorticoid inhibitory feedback (Herman 1993). For example, corticosterone implants into the medial preoptic area of rats inhibit the HPA axis responses to restraint stress (Viau & Meaney 1996). In addition, ventromedial hypothalamic lesions decrease the ability of low doses of corticosterone to inhibit basal ACTH secretion (Suemaru *et al.* 1995). Ablations of the arcuate nucleus, medial preoptic area, ventromedial nucleus or suprachiasmatic nucleus increase basal ACTH or glucocorticoid secretion and the magnitude and duration of the stress response (Viau & Meaney 1991, Buijis *et al.* 1993, Larsen *et al.* 1994).

Glucocorticoids also regulate further activity of the HPA axis by feeding back to extrahypothalamic regions of the brain including the hippocampus. An overall inhibitory influence of the hippocampus on HPA activity is supported by lesion studies in the rat and primate (Sapolsky *et al.*, 1984, Jacobson and Sapolsky, 1991, Sapolsky *et al.* 1991, Herman *et al.* 1995). These studies indicate that hippocampal lesion increases both the duration and magnitude of stress-induced glucocorticoid secretion while increasing the expression of CRH and AVP mRNA

in parvocellular PVN neurons. Stimulation of the hippocampus also results in decreased HPA activity in both rat and human (Jacobson and Sapolsky, 1991). Other structures in the limbic system, such as the prefrontal cortex or lateral septum also appear to inhibit the activity of the PVN. Lesion of these regions in the rat result in enhanced HPA responsiveness to acute stress such as restraint (Dobrakovova *et al.* 1982, Diorio *et al.* 1993). The effect of hippocampal manipulation on the HPA axis does vary both during the day and after stress suggesting a modulating role for glucocorticoids (DeKloet *et al.* 1998). The hippocampus exhibits greater glucocorticoid binding, and levels of glucocorticoid receptors (both high affinity mineralcorticoid (MR) and low affinity glucocorticoid (GR) receptors) than any other brain structure (Herman 1993, Jacobson and Sapolsky, 1991). Hippocampal MR activation maintains hippocampal excitability and through transsynaptic inhibitory projections to the PVN inhibits HPA axis basal activity (De Kloet *et al.* 1998). Conversely elevated glucocorticoid concentrations activate hippocampal GR receptors which suppress hippocampal output resulting in disinhibition of PVN neurons (De Kloet *et al.* 1998).

The CRH containing neurons of the PVN and the pituitary corticotrophs represent the primary feedback sites for stress levels of the naturally occurring glucocorticoids (De Kloet *et al.* 1998). However, at lower glucocorticoid concentrations, the brain is the primary target of feedback regulation as the access of glucocorticoids to receptors in the anterior pituitary corticotroph is hampered by competitive binding to intracellular CBG-like molecules (De Kloet *et al.* 1975, 1977, 1998). In contrast, synthetic steroids such as dexamethasone do not bind CBG (De Kloet *et al.* 1975, 1977) and are relatively poor at penetrating the brain due to their exclusion by the mdrla P-glycoprotein component of the blood brain barrier (Schinkel *et al.* 1995, Meijer *et al.* 1998). Thus at low concentrations synthetic steroids such as dexamethasone act predominantly at the pituitary level to inhibit stress induced HPA axis activity (De Kloet *et al.* 1974, 1975). This would suggest that the pituitary might be less sensitive to direct inhibition by increased glucocorticoid concentrations than the higher brain centres but that it would be more sensitive to inhibition by dexamethasone.

However, it is important to emphasise that not all CRH neurones are inhibited by glucocorticoids. CRH gene expression is increased in the central nucleus of the amygdala and the BNST following exposure to elevated levels of glucocorticoids, a mechanism by which stress may increase fear and anxiety states (Schulkin *et al.* 1998).

Other non-glucocorticoid inhibitory feedback loops also function to inhibit activity of the HPA axis. An ultrashort inhibitory loop mediated by CRH has been reported to function within the PVN of the rat (Calogero *et al.* 1988). Administration of ovine CRH inhibited basal secretion of rat CRH and decreased the responsivity of the CRH neurone to stimulatory inputs (Calogero *et al.* 1988). However, more recent studies in the rat have suggested that CRH may also exert an ultrashort positive feedback regulation on its own biosynthesis within the PVN (Parkes *et al.* 1993). In this study, however, CRH was administered *icv* and may have been acting on CRH receptors in other brain regions such as the brainstem catecholaminergic cell groups. A hypothalamic POMC gene-derived peptide loop has also been described (Johnson *et al.* 1992)

The pro-opiomelanocortin (POMC) derived peptides including the opioids (e.g. β-endorphin) and ACTH also plays an important role in modulating the activity of the HPA axis. Within the hypothalamus, POMC-containing neurons are primarily located in the arcuate nucleus. These neurons project to the PVN and also receive innervation from CRH containing neurones providing a closed feedback loop. During stress, activation of the arcuate nucleus by CRH release from the PVN results in the release of POMC peptides from axons projecting back to the PVN (Sawchencko *et al.* 1982, Nikolarakis *et al.* 1986, Burns *et al.* 1989). These POMC peptides limit the response of the HPA axis to stress by suppressing the synthesis of CRH in the PVN (Chrousos 1992, Chrousos & Gold 1992). ACTH has been reported to exert negative feedback effects on basal secretion of CRH (Suda *et al.* 1986, 1987) and on its own secretion from the anterior pituitary (Boscaro *et al.* 1988).

Keller-Wood & Dallman (1984) proposed that glucocorticoid mediated negative feedback occurs over three distinct time domains. The actions of glucocorticoid

feedback were described as being fast (seconds to minutes), intermediate (minutes to hours), and slow (hours to days). These differences in timing are considered to reflect the different mechanisms utilised by glucocorticoids to inhibit further activity of the HPA axis. With increasing time and steroid exposure the inhibition of the HPA axis becomes increasingly profound (Abou-Samra *et al.* 1986). Fast and intermediate feedback decreases subsequent stress-induced activation of the HPA axis by inhibiting the stimulus-evoked release of CRH, AVP and ACTH (Keller-Wood & Dallman 1984). In addition, intermediate feedback appears to inhibit CRH synthesis, though ACTH synthesis remains unaffected (Keller-Wood & Dallman 1984). However, basal activity of the HPA axis is not inhibited in both the fast and intermediate feedback time domains (Dallman *et al.* 1987b). In contrast, slow feedback inhibits both basal and stress induced activation of the HPA axis by inhibiting CRH and AVP gene expression in the PVN, and of POMC gene expression in the pituitary corticotroph (Roberts *et al.* 1979, Keller-Wood & Dallman 1984, De Kloet 1991). The delay in inhibiting ACTH production during slow feedback may be caused by the high stability of the mRNA encoding POMC and not by the absence of direct inhibition of the transcription (Mol & Rijnberk 1997).

Dallman Yates (1969) first proposed that the rapidly occurring component of glucocorticoid feedback is rate sensitive. This rapid inhibition occurs during the period of increasing plasma glucocorticoid concentrations with the magnitude of inhibition being proportional to the rate of increase in plasma glucocorticoids (Abe & Critchlow 1977). The rapidity of the inhibitory effect suggests a mechanism that does not involve protein synthesis and therefore is most likely to be mediated by cell surface receptors for glucocorticoids, rather than intracellular receptors that directly effect gene expression (Whitnall 1993). The mechanism by which this rapid inhibition is induced is unknown but may involve changes in membrane excitability following glucocorticoid exposure. Evidence for this hypothesis comes from studies examining the Ca^{++} stimulated release of CRH from hypothalamic cells. Glucocorticoids inhibit the Ca^{++} stimulated release of CRH whereas depolarisation of the hypothalamic cell membranes with K^{+} prevents this inhibition suggesting that fast feedback acts to stabilise the cell

membrane and may involve Ca^{++} fluxes (Keller-Wood & Dallman 1984). A similar process of reduced responsiveness to stimulation may also occur in the pituitary corticotroph. Alexander *et al.* (1993) demonstrated in the horse that acute lowering of plasma cortisol by inhibition of adrenal synthesis results in an increased ratio of ACTH to CRH in pituitary venous blood before CRH concentrations start to rise. This would suggest that the first effect is the opposite of fast feedback and is mediated by increased sensitivity of the pituitary corticotroph to CRH. Fast feedback is generally quantified by measuring the suppression of ACTH following an infusion of hydrocortisone (Young *et al.* 1991).

Intermediate feedback appears to involve integrated control processes, since the level and duration of inhibition vary according to the type and intensity of the feedback signal (Keller-Wood & Dallman 1984). For example, in the dog intermediate feedback is determined by the mean change in plasma glucocorticoid concentrations over time (Keller-Wood 1989). The responsive mechanisms involved in glucocorticoid mediated intermediate feedback are as yet not fully understood. However, intermediate feedback does require the presence of a protein whose synthesis is glucocorticoid-dependent and may involve involve gene-mediated effects and intracellular signal transduction pathways. Intermediate feedback can be tested by the suppression of HPA function following dexamethasone treatment, a test that involves glucocorticoid receptors in the pituitary (Miller *et al.* 1992). Both fast and intermediate types of feedback are reported to be impaired in animals exposed to chronic stress (Young *et al.* 1990).

Slow feedback is responsible for the tonic suppression that occurs when glucocorticoid levels have been elevated for hours or days i.e. in pathological conditions or following pharmacological treatment particularly with potent glucocorticoids such as dexamethasone (Keller-Wood & Dallman 1984). Slow feedback involves the classical genomic steroid mechanism of action, inhibiting CRH, AVP and POMC gene expression resulting in suppression of basal as well as stress-induced activation of the HPA axis.

A successful adaptation to a stressor (e.g. appropriate behavioural adaptation modulated by steroid effects on higher brain functions) induces negative feedback mechanisms acting via the PVN and neural inhibitory circuits to override the excitatory extrahypothalamic influences on the HPA axis. If coping with stress fails then an imbalance may develop between glucocorticoid inhibitory feedback and drive from excitatory inputs to the HPA axis resulting in continued activation of the HPA axis (Vamvakopoulos & Chrousos 1993, De Kloet *et al.* 1998). Hypersecretion of endogenous glucocorticoids associated with stress may lead to the down regulation of glucocorticoid receptors, impairing feedback regulation mechanisms and facilitating continued hyper activity of the HPA axis (Sapolsky & Plotsky 1990). Impairment of the glucocorticoid-receptor mediated negative feedback axis is termed glucocorticoid resistance and is a frequently reported effect of chronic stress and a well established biological marker of human endogenous depression (Arana & Mossman 1988). For example rats subjected to inescapable shock exhibit resistance to the suppressive effect of dexamethasone on endogenous corticosterone concentrations indicating impaired glucocorticoid negative feedback (Zhukov 1993). However, not all individuals follow this pattern of elevated glucocorticoid concentrations and glucocorticoid resistance during chronic stress. In group housed rats, a subgroup of chronically stressed subordinate animals displays a reduced corticosterone response to restraint stress (Blanchard *et al.* 1993, Albeck *et al.* 1997). This reduced corticosterone response may result from enhanced glucocorticoid negative feedback inhibition (De Kloet *et al.* 1998). Alternatively a deficit in the CRH drive (Sternberg *et al.* 1989) or altered sympathetic outflow diminishing adrenal sensitivity to ACTH (Buijs *et al.* 1997) may be responsible for the reduced adrenocortical output.

In deer, dexamethasone has been reported to suppress endogenous cortisol secretion when used as a pre-treatment before ACTH challenge (Goddard *et al.*1994, Smith & Bubenik 1990) but its use as a diagnostic test of glucocorticoid receptor mediated negative feedback has not been investigated.

2.7.6 Endogenous rhythms in HPA axis activity and function

The dynamic patterns of HPA axis basal activity exhibit variations over time that form consistent rhythms. These rhythms can fluctuate within or about 24 h (ultradian and circadian rhythms, respectively) or can be of longer duration, with variations occurring on an annual basis (seasonal or circannual rhythms). The ultradian rhythm in HPA axis activity is characterised by the pulsatile secretion of all the HPA axis hormones including CRH, AVP, ACTH and glucocorticoids. This pulsatile pattern of secretion have been described in a variety of species including humans (Iranmanesh *et al.* 1990, Veldhuis *et al.* 1990), rats (Windle *et al.* 1998, Ixart *et al.* 1991), cattle (Ladewig & Schmidt 1989), sheep (Kennaway *et al.* 1981), horses (Redekopp *et al.* 1986, Livesey *et al.* 1988, Alexander *et al.* 1994), and deer (Monfort *et al.* 1993). Pulsatile secretion is a common feature of neuroendocrine systems (e.g. the hypothalamic-pituitary-gonadal axis) and is critical to long term activation and physiological regulation (Veldhuis & Pincus 1998). In the case of the HPA axis it is thought the pulsatile activity prevents down-regulation of the HPA axis while maintaining its ability to respond to stress (Monfort *et al.* 1993).

The baseline ultradian pattern of HPA axis activity depends on a relatively constant oscillation of the CRH, AVP and CRH/AVP neurons of the PVN with the superimposition of several constitutive inputs, expressed primarily as changes in amplitude as well as possibly synchronisation of CRH and AVP pulses (Chrousos 1998). These inputs include positive circadian input(s) from one or more pacemakers (e.g. the suprachiasmatic nucleus (Hermes et al. 1991)), tonic positive input from the locus caeruleus/noradrenaline (LC/NA) and dopaminergic mesocorticolimbic systems, tonic negative input from the hippocampus and prefrontal cortex and negative feedback input from the arcuate nucleus-POMC-peptidergic system and glucocorticoids (Chrousos 1998). As CRH and AVP act synergistically at the pituitary corticotroph to stimulate ACTH secretion, both the amplitude and synchrony of their release may contribute to the amplitude of the ACTH pulse released. The release of ACTH from the anterior pituitary also follows a pulsatile ultradian rhythm of secretion, as demonstrated in the pituitary

venous effluent of the horse (Redekopp *et al.* 1986, Livesey *et al.* 1988, Alexander *et al.* 1994).

The ultradian rhythm in activity exhibited by the PVN and pituitary is also seen in the pattern of glucocorticoid release from the adrenal cortex. Pulsatile release of glucocorticoids has been demonstrated in a number of species including humans (Veldhuis *et al.* 1990), rats (Windle *et al.* 1998), cattle (Ladewig & Schmidt 1989), sheep (Kennaway *et al.* 1981), horses (Alexander *et al.* 1994), and deer (Monfort *et al.* 1993). The frequency of glucocorticoid pulses in the circulation is relatively constant in humans (Veldhuis *et al.* 1990) and rats (Windle *et al.* 1998). In these species, increases in glucocorticoid concentrations during the circadian peak or in response to stress appear to be due to changes in the pulse amplitude rather than increased pulse frequency (Figure 2.19) (Veldhuis *et al.* 1990, Windle *et al.* 1998). In deer, episodic secretion of cortisol, the predominant glucocorticoid (van Mourik *et al.* 1985, Smith & Bubenik 1990), has been shown in tame Eld's deer (*Cervus eldi thamin*) housed indoors (Monfort *et al.* 1993) though no information is available on cortisol secretory dynamics in red deer.

The generation of the stress-related increase in the activity of the HPA axis also depends on the relatively constant ultradian oscillation of the parvocellular CRH, AVP, and CRH/AVP neurons, and the superimposition of stress-related inputs, resulting in amplitude increases (Figure 2.19) (Chrousos & Gold 1992, Chrousos 1998). A direct relationship may exist between the pulsatile activity of the HPA axis and the magnitude of the response to acute stress. Windle *et al.* (1998) demonstrated that rats exposed to a short duration white noise stress exhibited a smaller response when the stimulus coincided with a declining phase compared with a rising (secretory) phase of a basal corticosterone pulse.

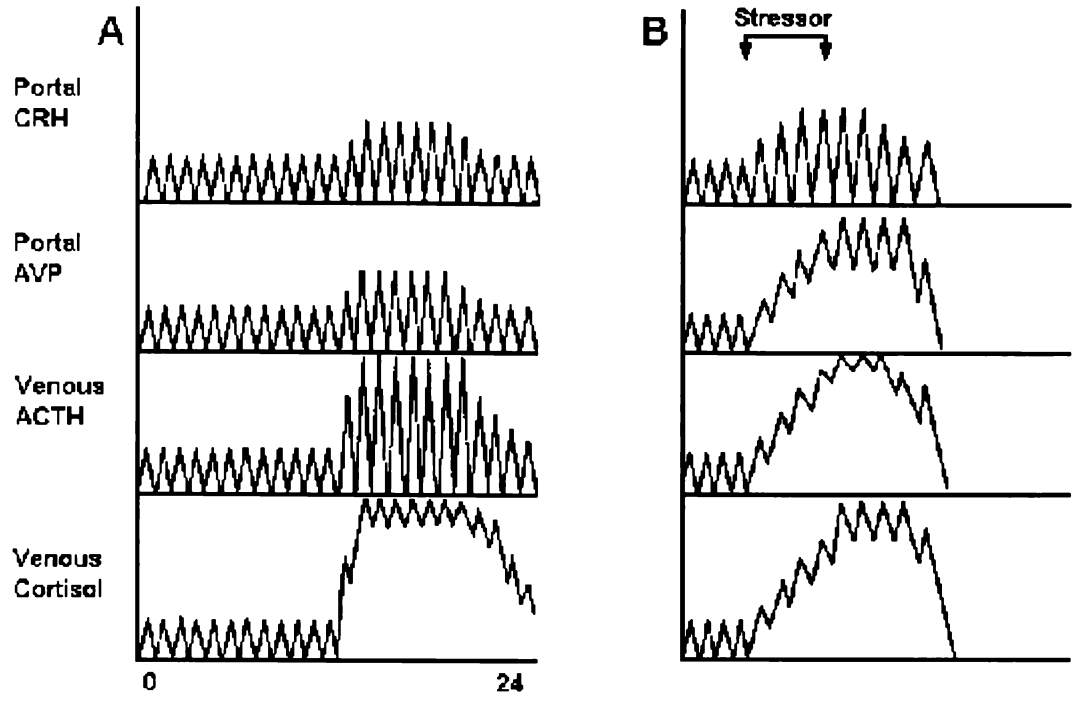


Figure 2.19 Representation of the secretory episodes of CRH and AVP in the hypophyseal portal system and of the resultant ACTH and cortisol concentrations in the systemic circulation. Note the relatively constant ultradian frequency and increasing circadian (A) and stress related (B) amplitude of the CRH, AVP, ACTH and cortisol pulses (adapted from Chrousos 1998).

Circadian rhythms in HPA axis activity have been observed in most mammals including humans (Weitzman *et al.* 1971) and rodents (Keller-Wood & Dallman 1984), with peak concentrations preceding the activity phase of the daily cycle. The circadian rhythm in HPA axis activity is generated in mammals by the activity of the suprachiasmatic nuclei. Direct inputs from the hypothalamic suprachiasmatic nucleus to the PVN have been described and are implicated in regulating the circadian rhythm in basal activity of the HPA axis (Berk & Finkelstein 1981). Inputs from the suprachiasmatic nucleus entrain the circadian rhythm in basal activity of the HPA axis to the light-dark cycle (Dallman *et al.* 1987b). Lesions of the suprachiasmatic nucleus diminish (Abe *et al.* 1979), or abolish (Raisman & Brown-Grant 1977) the circadian rhythm in HPA axis activity in the rat. In addition both feeding (Saito *et al.* 1989) and activity/sleep wake cycles (Born *et al.* 1997) also play a major role in entraining the circadian

rhythm. In domestic animals such as the horse (Irvine & Alexander 1994), pig (Janssens *et al.* 1995), sheep (Fulkerson & Tang, 1979) and cattle (Thun *et al.* 1981) the occurrence of circadian variations is generally acknowledged. However, in ruminants circadian rhythms are typically of low amplitude (e.g. cattle, 1-1.4 ng/ml, Thun *et al.* 1981, Lefcourt *et al.* 1993). At pasture, ruminants such as sheep, cattle and deer graze and rest in bouts throughout the day and night (Gates & Hudson 1983, Kilgour & Dalton 1984). Thus, they may not have the same cues available for entrainment of a circadian rhythm compared with non-ruminants or ruminants maintained indoors on fixed feeding schedules. For example, Simonetta *et al.* (1991) demonstrated that sheep held indoors and feed once a day exhibited a significant circadian rhythm in plasma cortisol whereas sheep feed at multiple times during the day and night showed no significant rhythm in cortisol.

There is no evidence from previous studies of deer for a circadian rhythm in HPA axis activity (white-tailed deer (*Odocoileus virginianus*), Bubenik *et al.* 1983; rusa deer (*Cervus timorensis*), van Mourik & Stelmasiak 1984a; Eld's deer, Monfort *et al.* 1993). However, in these studies the deer were subjected to either handling or indoor housing, factors known to attenuate circadian rhythms in other species (Irvine & Alexander 1994).

Apart from the relatively short term changes seen in the ultradian and circadian rhythms, HPA axis activity may exhibit changes of longer duration. Such changes can occur with ageing, during the oestrus cycle, during pregnancy and around the time of parturition. Seasonal changes in activity and responsiveness of the HPA axis have also been reported in a number of species including humans (Walker *et al.*1997), primates (Schiml *et al.* 1996), rodents (Boswell *et al.* 1994) and fish (McLeese *et al.* 1994). Evidence for a seasonal rhythm in adrenal activity or responsiveness in deer varies between species and studies, with seasonal changes reported in white-tailed deer (Bubenik *et al.* 1983), reindeer (*Rangifer tarandus*) (Nilssen *et al.* 1985), axis deer (*Axis axis*) (Chapple *et al.* 1991) and red deer (Suttie *et al.* 1995, Cassidy 1996) but not in Eld's deer (Monfort *et al.* 1993) or in another study on axis deer (Bubenik & Brown 1989).

2.7.7 Modifying factors in the HPA axis response to stress

The magnitude of the HPA axis response to stress depends on the inherent and acquired features of the individual, on its previous experience with the actual stressor, and on the qualitative properties of the stressor including the type (physical or emotional), strength, and duration (acute, chronic)(Johnson *et al.* 1992, Ladewig *et al.* 1993). These modifying influences include variables such as, time of day, season, age, gender, physiological condition, social factors, inherited or acquired individual characteristics, and strain or species differences. In this respect, the stress response is highly flexible and can vary considerably between individuals.

2.7.7.1 Individual differences

A number of features pertaining to the individual appear important in determining what constitutes a stressor and the type of adaptive response and the magnitude and duration of that response. Differences between individuals in their behavioural and physiological response to stress appear to be consistent. For example in pigs, individual differences have been observed in the adrenal cortex response to ACTH challenge and in behavioural activity in their home pen and during an open field test (Hennessy *et al.* 1988, von Borell & Ladewig 1989, 1992). These responses were consistent over repeated tests and the physiology and behaviour were correlated. Pigs with higher adrenal responsiveness to ACTH exhibited more locomotion and vocalisations during the open field test and were less active in their home pens than pigs with low adrenal responsiveness. Consistent individual differences in physiological and behavioural responses to stress appear to be related to genetic constitution, as well as to early life experiences (Suomi 1987, Johnson *et al.* 1992).

2.7.7.1.1 Genetic differences

There are marked differences in the stress response between different species, breeds and strains as well as individuals. Breed differences have been found in avoidance and escape behaviours in chickens (Craig *et al.* 1983) and fear of humans in sheep (Le Neindre *et al.* 1994). When such breed or individual

differences exist in a common environment, they are likely to be under some degree of genetic control. For example, about 2/3 of reliable variance measured in human complex behaviours such as personality traits are due to genetic influences (Bouchard 1994). Molecular genetic techniques have been used to identify genes involved in the variation in stress response. For example in pigs, a gene responsible for the porcine stress syndrome has been identified (Rempel *et al.* 1993).

2.7.7.1.2 Early development and ageing

A variety of aversive experiences *in utero* or in the neonatal period have been shown to have enduring effects upon emotional and neuroendocrine reactivity and individual patterns of stress susceptibility in later life. Fride *et al.* (1986) demonstrated that rats exposed to noise or light *in utero* develop a fearful temperament and increased corticosteroid responses to stress in later life. Similar results are seen in the offspring of pregnant rats exposed to immobilisation stress (Maccari *et al.* 1995). Levine (1957) showed that "neonatal handling" (human handling of newborn rats for 15 min daily during the first few weeks of life) actually decreased glucocorticoid responses to stress in later life. However rats handled at later points in time did not produce the same changes. Liu *et al.* (1997) was able to demonstrate that the increased levels of licking and grooming induced by the return of the rat pups after neonatal handling caused these changes. Handled pups and offspring from mothers who naturally produced high rate of licking and grooming had lower glucocorticoid responses to stress, more hippocampal glucocorticoid receptors and lower hypothalamic CRH mRNA levels in later life than offspring from mothers with lower rates of licking and grooming. This effect persists throughout the life cycle and influences age related cognitive decline (Meaney *et al.* 1988).

Individual patterns of stress susceptibility also change during ageing. Aged animals have an impaired ability to terminate the stress response (Sapolsky *et al.* 1983). It has been suggested that cortisol hypersecretion in aged animals is due to degenerative changes within the ageing brain and loss of sensitivity to glucocorticoid mediated feedback inhibition. These effects are seen primarily in

the ageing hippocampal region of the limbic system, a region associated with glucocorticoid mediated feedback inhibition of the HPA axis. In the ageing brain this region exhibits neuronal loss as well as a reduction (approx. 50 %) in glucocorticoid receptors, mainly type I (mineralocorticoid receptors) though some type II (glucocorticoid recptors) are also lost (Sapolsky *et al.* 1986). It appears that cumulative exposure to increased glucocorticoid concentrations over the lifespan of an animal might mediate hippocampal neuron death (Landfield *et al.* 1981). This ageing process can be accelerated by exposure to chronic stress and pharmacological doses of glucocorticoids (Sapolsky *et al.* 1986). Interestingly, treating elderly animals with antidepressants upregulates glucocorticoid receptor expression, thereby reversing aging-induced cognitive decline (Koenig 1999).

2.7.7.1.3 Sexual dimorphism in HPA axis activity and function

Sexual dimorphism in the response of the HPA axis to physical and psychological stress has been reported in a number of species (reviewed in Handa et al. 1994a, Vamvakopoulos & Chrousos 1994). Compared to males, females generally have higher basal levels of plasma glucocorticoids, greater adrenal responsiveness to ACTH and a higher and more prolonged elevation of glucocorticoids in response to stress while ovarectimised females and castrated males are intermediate in response (Gaillard & Spinedi 1998, Handa et al. 1994a). The hypothalamic-pituitary-gonadal (HPG) axis has been shown to play a critical role in the development of this sexual dimorphism with estrogens enhancing and androgens diminishing HPA axis basal activity and responsiveness to stress (Critchlow et al. 1963, Handa et al. 1994a).

In both males and females gonadal steroids modulate the activity of the HPA axis at multiple levels of the axis. In the female, estrogens directly effect CRH gene expression (Vamvakopoulos & Chrousos 1993), enhancing CRH levels within the paraventricular nucleus (PVN) (Haas & George 1988). Glucocorticoid receptor mediated negative feedback of HPA axis activity is also impaired by estrogens (Burgess & Handa 1992) with glucocorticoid receptor levels being estrogen dependent (Turner 1990). In addition to these centrally mediated effects estrogen increases cortisol production from the adrenal (Kitay 1963) and anterior pituitary

responsiveness to CRH (Coyne & Kitay 1971). Estrogens also increase plasma corticosteroid-binding capacity by increasing the hepatic synthesis of corticosteroid-binding globulin (CBG), the function of which is thought to regulate the bioavailability of circulating glucocorticoids to target tissues (Coe et al. 1986).

In males, the removal of gonadal steroids by castration will increase the ACTH and glucocorticoid response to physical and psychological stressors as well as increasing levels of CRH and numbers of CRH containing cells within the PVN, however, at least 10 days is required following castration before increases are apparent in the rat (Bingaman et al. 1994, Handa et al. 1994a). Androgens are believed to indirectly inhibit CRH levels within the PVN by binding to regions of the brain rich in androgen receptors (AR) which project to the PVN and influence HPA axis activity (e.g. hippocampus, medial preoptic nucleus, bed nucleus of the stria terminalis, and lateral septum) (Handa et al. 1994a). However, castration is not accompanied by a decline in anterior pituitary responsiveness to CRH in rats (Handa et al. 1994a).

Androgens may also increase the ability of glucocorticoids to inhibit HPA axis activity. Viau & Meaney (1996) reported that high physiological levels of testosterone increase glucocorticoid receptor binding in the medial preoptic area (MPOA), with no effect on mineralocorticoid receptor binding or on glucocorticoid receptor binding in other regions. They also reported that testosterone administration decreased arginine vasopressin (AVP) levels in median eminence, which was correlated with the magnitude of the ACTH response to restraint stress. They concluded that the MPOA is a critical site for androgen inhibition of HPA axis responsiveness and that this effect is via the inhibition of hypophysial AVP (Viau & Meaney 1996).

Sexual dimorphism of the HPA axis is also seen at the level of the adrenal gland with the female or castrate adrenal cortex been more responsive to ACTH than that of the intact male in terms of glucocortiocid production (Bass et al. 1982, Kitay 1961, 1963, Verkerk & Macmillan 1997). Androgens inhibit glucocorticoid production directly by influencing steroidogenic pathways involved in the

synthesis of glucocorticoids in the adrenal cortex (Gaskin & Kitay 1971, Hornsby 1982; Miller 1988).

Since plasma concentrations of glucocorticoids represent a balance between adrenal secretion and the rate of clearance from the circulation, regulation of the metabolic clearance rate of cortisol may constitute another important adaptive mechanism. Sexual dimorphism in the metabolic clearance rate of cortisol has been reported in several species including the guinea-pig (El Hani et al. 1980, Greiner et al. 1976) with higher rates of clearance in males than in females or castrates (El Hani et al. 1980), with testosterone treatment of female guinea-pigs resulting in a increase in the metabolic clearance rate of cortisol (El Hani et al. 1980).

Furthermore, the cortisol binding capacity of plasma contributes to the regulation of cortisol concentrations by regulating the bioavailability of circulating glucocorticoids to target tissues (Coe et al. 1986). The cortisol binding capacity of plasma is largely determined by the concentrations of corticosteroid-binding globulin (CBG) which binds cortisol with much higher affinity and specificity than albumin and is the major determinant of cortisol bioavailability (Hammond 1997). An inverse relationship appears to exist between androgens and CBG binding capacity, castration of male rats leads to a 40-50% increase in CBG concentrations which return to normal with testosterone treatment (Mataradze et al 1992) while the males of many seasonally breeding species exhibit an androgen dependent fall in CBG concentration during the breeding season (Bradley & Stoddart 1992, McDonald et al. 1981). Androgens may reduce the corticosteroid-binding capacity of plasma by suppressing the hepatic synthesis of CBG (Gala & Westphal, 1965) or by competing with cortisol for binding sites on corticosteroid carrier proteins such as CBG and albumin (Ballard, 1979, Hammond, 1990) effectively increasing unbound concentrations of cortisol.

2.7.7.2 Characteristics of the stressor

The adaptive response to stress is also dependent on the quality (e.g. physical or psychological), strength, duration (e.g. acute or chronic), predictability and

controllability of the stressor (Johnson *et al.* 1992). Stressors of similar nature and magnitude may cause completely different levels of physiological reactions dependent on the experiences of the animals (Jensen & Toates 1997). The response to repeated stressors depends upon a variety of factors such as intensity, duration, number of repetitions, and frequency of the application (Natelson *et al.* 1988). With repeated exposure to less intensive stressors, the stress response declines (habituation), whereas with more intensive or painful stressors the stress response increases upon repeated exposure (sensitisation) (Konarsky *et al.* 1990, Pitman *et al.* 1990).

Weiss (1971) demonstrated the importance of three key psychological key components, predictability, controllability and feedback in determinig the magnitude of the response to stress. When rats where exposed to tail shock, HPA axis activation and ulceration's were much smaller if the shocks were pre-signalled and even less if animals could postpone or shorten the duration of the shock by turning a wheel. When the rats were offered independent feedback to certify that their control attempts were successful, their physiological reactions were as small as controls that never received any shock. The importance of these modifying factors implies that the impact of a stressor is not only dependent on stressor intensity but also defined by the situation and, most importantly, by the way it is experienced by the individual.

2.7.7.2.1 Duration of the stressor

In acute stress situations, increased plasma cortisol responses are associated with more intensive handling procedures (Carragher et al. 1997) and may reflect the animal's perception of the severity of the stressor imposed. The effect on the HPA axis of repeated or chronic exposure to a stressor is, however, not well understood. Different measures of the activity of the HPA axis are increased, unaffected or depressed by exposure to chronic stressors (Jensen et al. 1996a, Rushen 1991). Habituation of the glucocorticoids response to acute or novel stressful stimuli may occur as the stimulus is repeated or becomes more chronic. However, this is not always the case as glucocorticoid concentrations may remain

elevated (Roman-Ponce et al 1981) normalise (Ladewig & Smidt 1989) or become depressed (Rhynes & Ewing 1973) with continued exposure to chronic stress.

Interpretation of changes in basal cortisol concentration may be further confounded by the observation that chronic stress can lower the cortisol binding capacity of plasma and hence increase the proportion of biologically active free cortisol present in circulation (Alexander & Irvine 1998, Blanchard et al.1993, Kattesh et al. 1980, Spencer et al. 1996). The function of this stress-induced fall in binding is unclear but may serve to increase free glucocorticoid concentrations thereby increasing their effect on target tissues (Fleshner et al 1995).

In addition to changing total and free glucocorticoid concentrations, chronic stress has been reported to influence how specific elements of the axis function including responsiveness of the adrenal cortex to ACTH (Friend et al. 1977, 1979, Ladewig & Smidt 1989) and the pituitary corticotrophs to CRH (Alexander et al. 1996, Odio & Brodish 1990). Reduced pituitary responsiveness to CRH is characteristic of a number of human psychiatric disorders such as depression, panic disorder, anorexia nervosa and post-traumatic stress disorder (Smith & Hammond 1989). Chronic social stress in horses has been shown to reduce pituitary responsiveness (ACTH secretion) to an exogenous CRH challenge and to increase pituitary venous blood concentrations of CRH in animals at rest (Alexander et al. 1996). Responses to CRH challenge have not been reported for red deer, however, CRH is a potent stimulator of ACTH secretion by cultured pituitary corticotrophs from fallow deer (*Dama dama*)(Willard et al. 1995).

Impairment of the glucocorticoid-receptor mediated negative feedback of HPA axis activity is another frequently reported effect of chronic stress and has been well established as a biological marker of human endogenous depression (Arana & Mossman 1988). Hypersecretion of endogenous cortisol associated with stress may lead to the down regulation of glucocorticoid receptors, impairing feedback regulation mechanisms and facilitating continued hyper activity of the HPA axis (Sapolsky & Plotsky 1990). Resistance to the suppressive effect of dexamethasone, a synthetic glucocorticoid, has been shown in rats subjected to inescapable shock (Zhukov 1993) and reflects the impairment of glucocorticoid

negative feedback mechanisms. In deer, dexamethasone has been reported to suppress endogenous cortisol secretion when used as a pre-treatment before ACTH challenge (Goddard et al 1994, Smith & Bubenik 1990) but its use as a diagnostic test of glucocorticoid receptor mediated negative feedback has not previously been reported.

Variability in the responses of HPA axis parameters to chronic stress may be due to specific species differences, the use of experimental subjects of different sex and reproductive status or to differences in experimental protocols, including differing sampling times and methods. The quantitative and qualitative properties of the stressor may also influence responses. However, Jensen et al. (1996a) have proposed that rather than being a permanent state, the response to chronic stress may alter during the time course of exposure to the repeated stressor with changes in response at different levels of the HPA axis and related systems such as behaviour and the CNS. These changes are characterised by an initial activation of the HPA axis followed by biochemical adjustments that result in an apparent normalisation or even suppression of the activity in the HPA axis over time (Jensen et al. 1996a).

2.7.8 Monitoring the HPA axis

Changes in the activity and functioning of the hypothalamic-pituitary-adrenal (HPA) axis are routinely used to quantify an animal's response to stress. However, the stress associated with handling and restraint of animals with traditional blood sampling techniques can in itself cause activation of the HPA axis, thereby confounding such measurements (Seal *et al.* 1972, Hattingh *et al.* 1988). This effect is further exacerbated in wild or semi-domesticated animals or in species with flighty natures such as red deer.

A variety of techniques have been developed in an effort to minimise the stress imposed on the animal during sample collection. These include rendering the animal unresponsive to the effects of handling by shooting (Hattingh *et al.* 1984, Smith & Dobson 1990) or sedation (Monfort *et al.* 1993), habituation to the blood sampling procedure (Bubenik *et al.* 1983) and the use of techniques which reduce

or eliminate the degree of handling. These include measurement of corticosteroids in substances other than blood (eg. saliva, Cook & Jacobson 1995; milk, Verkerk *et al.* 1998; urine and faeces, Palme *et al.* 1997), the use of indoor remote catheter systems (Ladewig & Stribrny 1988, Monfort *et al.* 1993), or remote portable blood sampling devices (Farrell *et al.* 1970, Bubenik & Bubenik 1979, Hattingh *et al.* 1988, Mayes *et al.* 1988, Stephan & Cybik 1989, Goddard *et al.* 1994,1998, Ingram *et al.* 1994, 1997, 1999, Ferre *et al.* 1998). These existing procedures, however, have limitations when it comes to assessing the activity and functioning of the HPA axis. Shooting animals affords only limited information on cortisol concentrations at the time of death with no opportunity to follow changes over time in the same individual. Measurements obtained from sedated animals may vary due to the difficulty in maintaining consistent levels of sedation over time and between individuals (Monfort *et al.*, 1993) while some sedatives (eg. Xylazine hydrochloride, Chao *et al.*, 1984) may affect the HPA axis directly.

A process of taming and habituating an animal to the blood sampling procedure may result in animals with atypical responses when compared with those of wild or semi-domesticated con-specifics. This is of particular importance when handling is an integral component of the stressor being assessed (e.g. shearing or transport of live animals).

Collection of saliva or milk samples from lactating females, though less invasive than blood sampling still requires some degree of handling. Though collection of urine or faeces can be achieved without handling, the integrated estimates of HPA axis activity obtained with this technique lack the temporal precision for detecting fluctuations in circulating glucocorticoids that are important in assessing responses to acute stress, or in tests of HPA axis function.

The use of static remote catheter systems for obtaining blood from undisturbed animals also involves an extended process of habituation to indoor housing and in some cases restraint of the animal, which can place limitations on the type of animal and the stressor that can be evaluated.

The use of remote blood sampling systems can overcome many of these limitations. Such a technique can be applied in the assessment of a wide range of stressors and removes the requirement for repeated handling and the need for habituation to indoor housing, though some degree of habituation to carrying the sampling equipment may be required. The relatively large size of remote blood sampling systems and the requirement in most systems to store the blood sample on the animal until retrieval does, however, impose limitations upon the size of animal that can be remotely sampled as well as restricting any measurement to compounds that are relatively stable over time in whole blood. However, remote blood sampling systems do lend themselves to the monitoring of glucocorticoid concentrations, which are relatively stable in whole blood for several days (Reimers *et al.* 1983), and the sampling of large, flighty, free ranging animals (such as red deer).

To accurately quantify ultradian and circadian rhythms in HPA axis basal activity, a remote blood sampling system would need to collect samples over 24h at a frequency that can accurately measure glucocorticoid pulsatility (sampling at least every 20min, Monfort *et al.*, 1993). To remotely measure changes in HPA axis function a device capable of administering, by remote bolus infusion, ACTH or CRH challenges and monitoring the subsequent adrenocortical response would be ideal. The DracPac remote blood sampling device was therefore developed with these specifications in mind (see Chapter 3, 4 and 5). Such a system would mitigate the confounding effects of blood sampling stress associated with manual methods of blood collection, thereby allowing the true effects of stress to be revealed.

2.8 THESIS OUTLINE

A number of farm animal husbandry practices result in stress and can potentially affect the welfare of livestock. When evaluating the welfare impacts of husbandry practices or when comparing alternative procedures, a quantifiable index of the degree of stress experienced by the animal is required. These indices may include behavioural, autonomic and endocrine changes that occur in response to a wide

range of intrinsic or extrinsic stressors. A number of studies investigating deer farming practices have reported details regarding behavioural (e.g. Diverio *et al.* 1996a, Pollard *et al.* 1998), immunological (e.g. Griffin *et al.* 1991, Hibma & Griffin 1994, Thomson *et al.* 1994, Hanlon *et al.* 1995) and cardiovascular (e.g. Diverio *et al.* 1996b, Pollard *et al.* 1992, 1993, Price *et al.* 1993, Carragher *et al.* 1997, Matthews *et al.* 1994) changes in response to apparently stressful procedures. However, it is generally accepted that many aspects of the stress response are mediated or in some way regulated by the HPA axis. Thus, monitoring stress-induced changes in HPA axis activity may offer quantifiable measures of the degree of stress experienced by an individual, an important criteria in the assessment of the welfare status of an animal. In fact changes in the activity and function of the hypothalamic-pituitary-adrenal (HPA) axis are routinely used to assess welfare in farm animals (Barnett & Hemsworth 1990, Fraser & Broom 1990).

There is little information pertaining to the basal activity and functioning of the HPA axis and how these may change in response to stress in red deer (Ingram *et al.* 1994, 1997, 1999, Carragher *et al.* 1997, Waas *et al.* 1997, 1999). This lack of information may reflect the difficulties in obtaining meaningful measurements of HPA axis activity and function in this species. It has been demonstrated that such measurements are readily confounded by the stress associated with handling and restraint of animals using traditional blood sampling techniques (Seal *et al.* 1972, Hattingh *et al.* 1988). This effect is further exacerbated in wild or semi-domesticated animals or in species with flighty natures such as red deer (Ingram *et al.* 1994, 1997, 1999, Carragher *et al.* 1997). To avoid the confounding effects this handling may have on basal activity of the HPA axis in red deer and its response to stressors of interest, we undertook to develop a remote blood-sampling device capable of obtaining blood samples from undisturbed animals at pasture.

In Chapter 3 we investigated the validity of using remote blood sampling methodology on free-ranging red deer. A number of potential factors which could affect the accuracy of measurements obtained from remotely collected blood samples were investigated. These included the stability of different analytes (e.g. cortisol) in whole blood during storage of the sample on the animal prior to

collection and processing. It was also important to establish whether animals would adapt to carrying the blood sampling equipment and how long the period of recovery would be after attachment of the equipment before values returned to baseline levels.

True baseline measurements of HPA axis activity are also important in the assessment of HPA axis function. The responsiveness of the adrenal cortex to exogenous adrenocorticotropic hormone infusion (ACTH challenge) is widely used as an index of adrenocortical function (Barnet & Hemsworth 1990). A number of studies have reported a link between altered adrenocortical responsiveness to ACTH challenge and stress (e.g. Friend *et al.* 1977, 1979, Ladewig & Schmidt 1989, Goddard *et al.* 1994). In red deer, a number of studies have reported the effects of ACTH challenge in physically restrained or sedated animals (e.g. Goddard *et al.* 1994, Jopson *et al.* 1990, Bubenik & Bartos 1993, Hanlon *et al.* 1994, Suttie *et al.* 1995). However, the stress associated with handling or the sedatives and differing levels of sedation makes interpretation of these responses difficult (Monfort *et al.* 1993). Chapter 4 describes the development of a remotely administered ACTH challenge test for red deer that allows adrenocortical responsiveness to be assessed in undisturbed animals at pasture thus eliminating the confounding effects of handling stress.

In addition to stress induced changes in HPA axis activity, most species exhibit endogenous variations over time in basal activity and responsiveness of the HPA axis. These variations can form a consistent rhythm which fluctuates within or about 24 h (ultradian and circadian rhythms, respectively) or can be of longer duration, with variations occurring on an annual basis (seasonal or circannual rhythms). Endogenous rhythms in HPA axis activity vary between species and are believed to effect the magnitude of the response to stress. Thus interpretation of a species HPA axis responses must take into consideration these endogenous variations before such measures can be used in the assessment of stress. Chapter 5 describes the quantification in the red deer stag of ultradian, circadian and seasonal rhythms in basal HPA axis activity. In addition, seasonal changes in adrenal and HPA axis responsiveness following challenges with ACTH and a standardised acute stressor were quantified.

The dramatic seasonal rhythm in the HPA axis activity and function observed in Chapter 5 followed closely that of the seasonal cycle in reproductive function of the red deer stag. Chapter 6 investigated the role of gonadal steroids in modulating the seasonal rhythm of HPA axis activity and function in the red deer stag by comparing basal activity and the functioning of components of the HPA axis in castrated and entire red deer stags during the breeding season. This included quantifying differences in response to ACTH, CRH and dexamethasone challenges. Other mechanisms involved in the regulation of the HPA axis and cortisol concentrations such as plasma steroid binding capacity (i.e. free cortisol concentrations) and the clearance rate of cortisol from circulation were also evaluated.

The response of the HPA axis to repeated or chronic stress is not well understood. In Chapter 7 we used a model of chronic social stress induced by repeated mixing of individual stags into unfamiliar groups to investigate the time course of effects of chronic social instability on behaviour and hypothalamic-pituitary-adrenal axis (HPA axis) activity and function, in mature red deer stags. Mixing of unfamiliar deer has been shown to elicit aggression and induce physiological responses typical of stress both in the short term (Pollard *et al.* 1993, Pollard & Littlejohn 1998) and when mixing is repeated over time (Hanlon *et al.* 1995).

Despite selective breeding for good temperament farmed red deer still maintain much of the flighty nature characteristic of their wild conspecifics. Previous studies have highlighted the behavioural and physiological reactivity of farmed red deer to stress (e.g. handling, Carragher *et al.* 1997; transport, Waas *et al.* 1997, 1999; indoor housing, Goddard *et al.* 1994, Hanlon *et al.* 1994, Pollard & Littlejohn 1998; social stress, Hanlon *et al.* 1995). The behavioural and physiological reactivity of deer and their susceptibility to stress-induced pathology highlights the need for quantifiable measures of stress in this species. The technique of remote blood sampling and its application in the studies described in the following chapters offer unique information on the use of a variety of measures of HPA axis activity and function in the assessment of stress in this species.

CHAPTER 3 A STRESS FREE BLOOD SAMPLING TECHNIQUE FOR FREE RANGING ANIMALS

3.1 ABSTRACT

Changes in various blood parameters can be used to assess the relative stressfulness of animal farming practices. In order to overcome confounding effects of stress inherent in standard methods of blood collection, a portable remote blood sampling device ("DracPac") has been developed and tested. Twelve heparinised blood samples can be taken from the jugular vein and stored in insulated packs on the animal. The stability of blood parameters taken from and stored on red deer was determined. Analyses of subsamples taken from a bulk initial sample over 12 hours showed that levels of cortisol, haematocrit and glucose did not significantly differ from initial values. In a second experiment stags were sampled remotely during restraint in a mechanical crush and subsequent recovery at pasture on 2 successive days. On both days cortisol, haematocrit, glucose and lactate levels were elevated during restraint and thereafter decreased significantly to reach levels substantially lower than previously reported for this species using standard methods of blood sampling. The development of this device permits reliable remote blood sampling of free ranging animals without the stress associated with manual blood sampling.

3.2 INTRODUCTION

Increasing concern about animal welfare has lead to attempts to evaluate the relative stressfulness of various farming practices. Changes in the levels of a

number of physiological and biochemical parameters in blood plasma are commonly used to quantify the degree of stress an animal experiences. Traditional blood sampling methods commonly involve some form of handling and restraint which, in many cases, can itself induce a stress response and confound interpretation of the measurements obtained (e.g. Seal *et al.* 1972, Hattingh *et al.* 1988).

Attempts to minimise the stress involved in blood sampling include: shooting undisturbed animals in paddocks (Smith & Dobson 1990); habituation to handling and the sampling procedure (Bubenik *et al.* 1983); the use of static remote systems on tame animals in pens (Ladewig & Stribrny 1988) and remote portable devices outdoors (Farrell *et al.* 1970, Bubenik & Bubenik 1979, Hattingh *et al.* 1988, Mayes *et al.* 1988, Stephan & Cybik 1989). These methods have limitations. Shooting does not allow repeat sampling; habituation and taming result in atypical animals; and although current remote systems overcome the confounding effects of handling, they are not commercially available.

Since remote systems appear to provide the optimal sampling procedure, particularly for application with large, flighty, free ranging animals (such as red deer), we undertook to develop such a device that was both versatile and reliable. The present study outlines 2 experiments carried out to this end. Thus, the stability of blood samples stored on animals, and the measurement of levels of stress parameters during handling and subsequent recovery of red deer in the field, were determined.

3.3 MATERIALS AND METHODS

3.3.1 Procedure for experiment 1

Two rising 2 yr red deer stags were restrained in a pneumatic deer crush and blood (50 ml) sampled from the jugular vein. The blood from each animal was then mixed with 3 ml heparinised saline (5000 IU/ml) and stored on an animal outdoors for 12 h in insulated packs containing icepacks. The samples were subsampled at 0, 0.5, 1.5, 3, 6, and 12 h. Whole blood was analysed for

haematocrit, and plasma was separated by centrifugation and stored at -20°C until analysed for cortisol, lactate, and glucose (see below). The temperature inside the insulated packs was also monitored using a temperature logging device (Delphi Temperature Logger, model 861, Temperature Logger Systems, Wellington N.Z.).

3.3.2 Procedure for experiment 2

Twelve rising 2 yr red deer stags were used; 6 were fitted with remote blood sampling devices (experimental animals), the remaining 6 were included to maintain normal group size. Experimental animals were sedated with Fentazin (1.2 mg xylazine/kg) and a double lumen catheter was inserted into the jugular vein (see Ladewig & Stribrny 1988). At the same time animals were fitted with a canvas backpack. The sedative was reversed with Yohimbine (0.25 mg/kg i.v.).

The following day (Day 1) the experimental animals were restrained in a pneumatic crush (Figure 3.1) and 10 ml of blood was withdrawn manually from the catheter (sample 1). The animals were then fitted with a battery powered remote blood sampling device (DracPac).

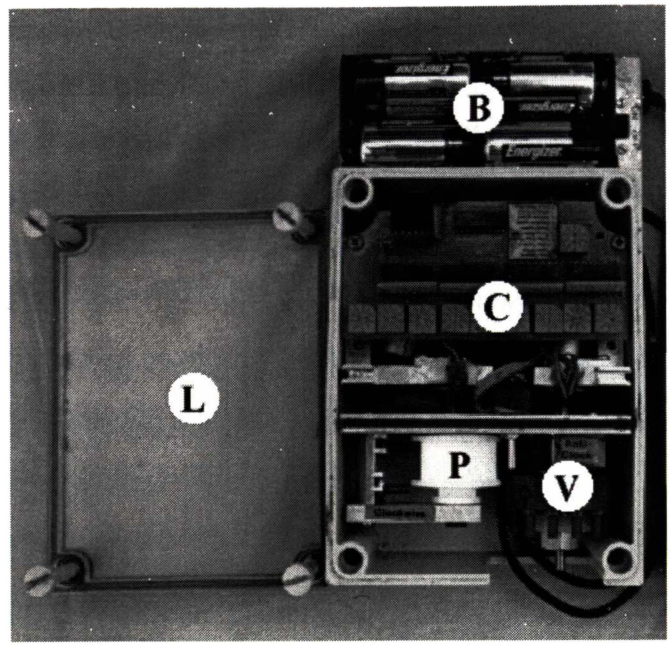
Figure 3.1 Red deer stag restrained in a pneumatic crush during the attachment of the DracPac remote blood sampling device

This device, developed jointly by the Animal Behaviour and Welfare Research Center and the Engineering Development Group, is shown in Figure 3.2 and consists of a small peristaltic pump that delivers heparinised saline (5000 IU/ml) down one lumen to the tip of the catheter where it mixes with blood continuously being drawn up the second lumen. The blood is then pumped through a 12-position rotary switching valve (Mayes *et al.* 1988) and collected in one of 12 separate PVC bags. The samples are stored on ice in an insulated pouch within the backpack. The pumping rate and sample duration are independently controlled by a microprocessor, and together determine sample volume. The device measures 150 x 110 x 60 mm, and weighs 1.3 kg.

The blood sampler was programmed to collect 11 continuous samples, each of 20 minutes duration. The animal was then released to pasture (Time 0; approximately 15 min after sample 1 was taken) (see Figure 3.3). Following collection of the last sample at pasture, the experimental animals were returned to the yards and the blood sampler and samples removed from the backpack. The procedure of fitting the sampler and obtaining the samples was repeated the next day (Day 2).

3.3.3 Blood Sample Analyses

Haematocrit was determined by centrifugation of whole blood in microcapillary tubes in duplicate. Plasma lactate and glucose were determined using Bohringer Mannheim kits on a Hitachi 717 random access analyzer according to manufacturers instructions. Plasma cortisol concentrations were measured in duplicate, and after extraction in ethyl acetate, by an ^{125}I radioimmunoassay method with PEG separation. All samples were measured in a single assay; intra-assay co-efficient of variation was 10.1%. Assay sensitivity was 0.33 ng/ml.



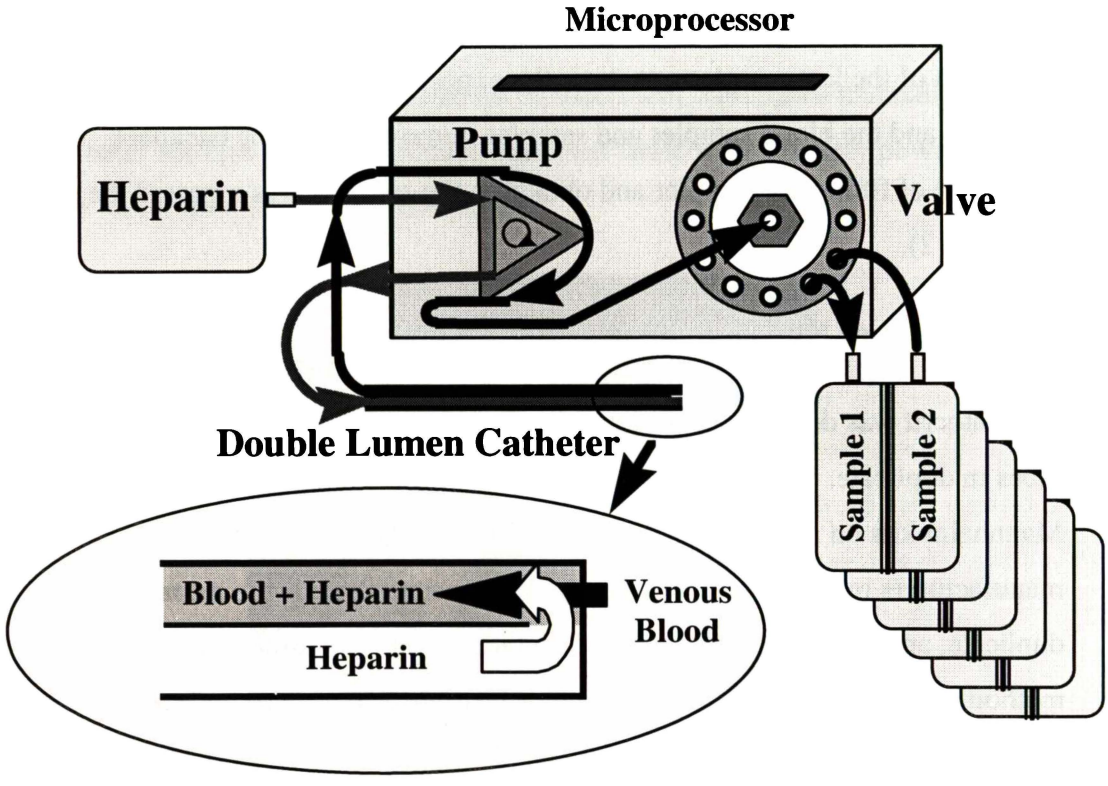


Figure 3.2 Photograph of the DracPac remote blood sampling device with the lid (L) removed showing the battery compartment (B), microprocessor and control unit (C), peristaltic pump (P) and the 12-position rotary switching valve (V). Below is a schematic representation of the DracPac device and the technique used to obtain blood samples (see text for explaination). The double lumen catheter (Cavafix Duo16/18G 32cm, Braun, Germany) is modified by removing 1-2 mm of the wall between the two lumina at the tip to facilitate optimal mixing of the outflowing heparin and inflowing blood (see Ladewig & Stribrny, 1988).

Figure 3.3 Stags at pasture carrying backpacks containing the DracPack remote blood sampling device.

3.3.4 Statistical Analysis

The data from Experiment 1 was subjected to linear regression analysis (Genstat, 1990) to determine trends. The data from Experiment 2 was analysed by orthogonal polynomial contrasts of the time profiles; least squares ANOVA (Genstat, 1990) was used to test for animal and day effects. Significance levels were set at $P<0.05$. Exponential functions were fitted to individual data on each day to describe their rate of change. Estimates of asymptotes were used to derive apparant basal values of the blood parameters. All values are presented as the mean ± S.E.M.

3.4 RESULTS

3.4.1 Experiment 1

The temperature inside the insulated blood pack remained below 4^0C over the 12 h experimental period. Figure 3.4 shows the percentage change from time zero for haematocrit and plasma cortisol, lactate and glucose, for both bulk samples. The levels of cortisol, haematocrit and glucose did not change significantly over time, whereas, lactate levels changed significantly with increasing storage time (P= 0.001) increasing by 12.8 % over 12 h.

3.4.2 Experiment 2

Jugular catheters were found to be patent in 5 animals on Day 1 and 3 animals on Day 2. Blood samplers were fitted to these animals and 99 % of expected samples were successfully collected.

The haematocrit, plasma cortisol, lactate and glucose levels during restraint and successive 20 min sampling intervals after release to pasture on days 1 and 2 are shown in Figure 3.5. Mean plasma cortisol concentrations were 56.5 ± 5.1 ng/ml during restraint and there was no significant difference between the levels on days 1 and 2. There was also no significant difference between cortisol levels in samples taken manually and the first samples taken with the blood sampler on each day. On both days cortisol levels decreased significantly with time after release to pasture. The rate of decrease was not significantly different (P=0.08) between the 2 days.

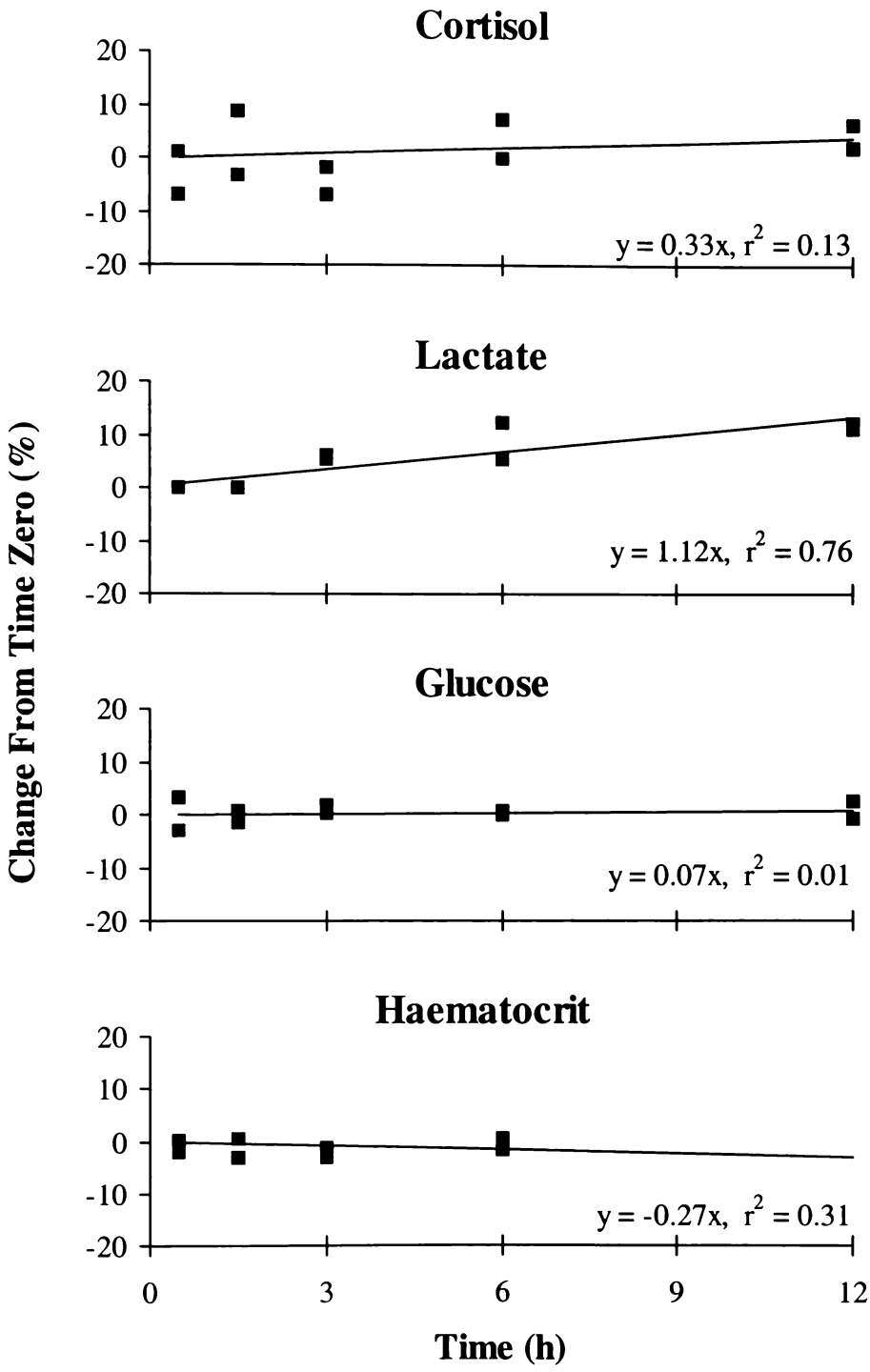


Figure 3.4 The effect of storage time on the percentage change in levels of cortisol, lactate, haematocrit and glucose relative to initial values (Time 0). All values for the samples from the two different animals are shown. The equations and lines for the least squares linear regression are also shown.

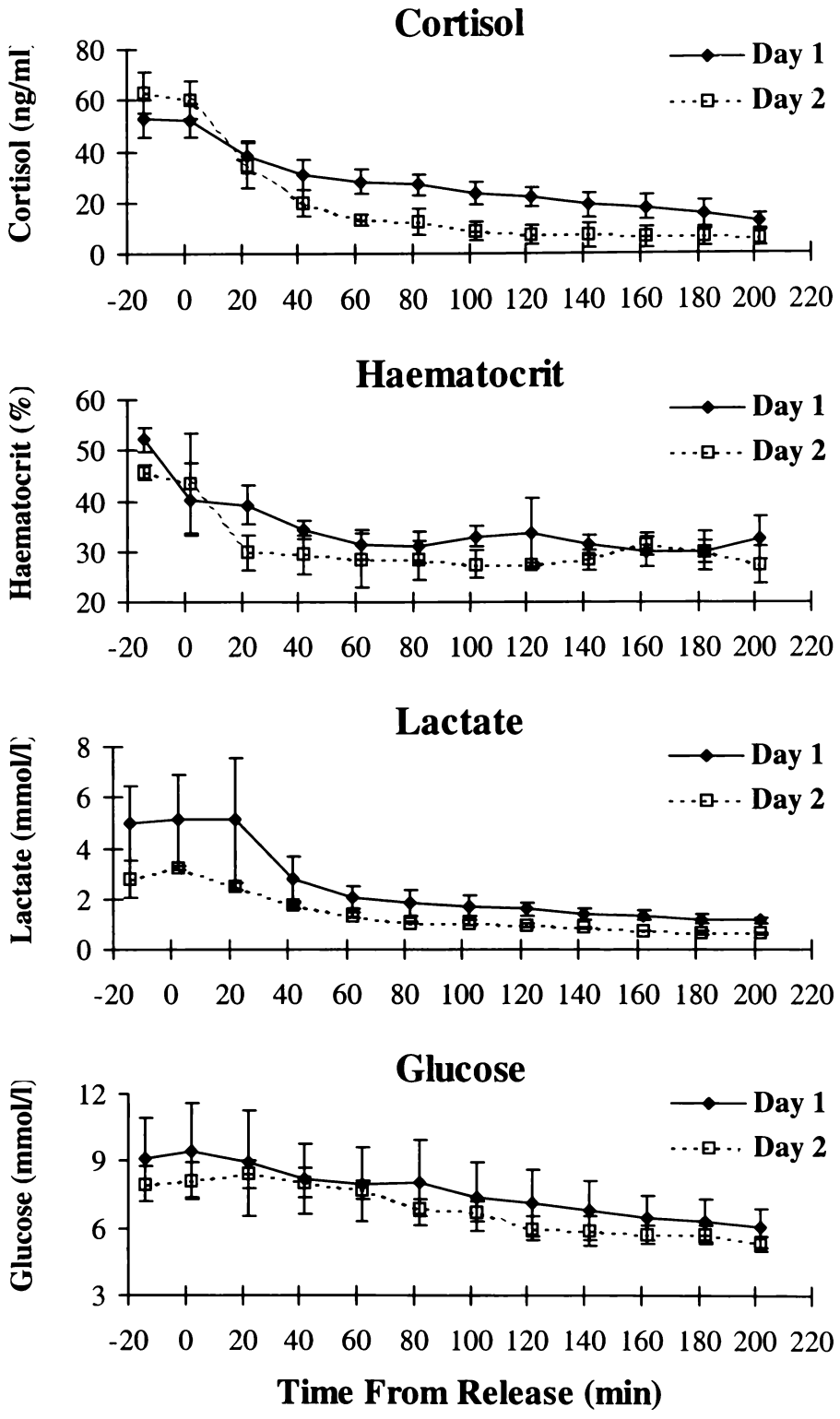


Figure 3.5 Levels of cortisol, lactate, haematocrit and glucose during restraint and following release to pasture (Time 0) on the two experimental days. The two values to the left of Time 0 on each plot are for the samples taken manually in the crush. Day 1 n = 5; Day 2 n = 3.

Plasma cortisol levels declined exponentially ($r^2 = 0.96$) after release of animals to pasture. Estimated basal levels (the mean asymptotic value for the individual exponential curves) and estimated recovery times (the mean time at which the fitted curve attained a value within 10% of the asymptotic value) are shown in Table 1 for days 1 and 2 combined. The estimated basal level for cortisol was 8.4 ng/ml with the estimated recovery time been 3.75 h after release.

Parameter	Estimated basal level*	Estimated recovery time**	r^2 value
Cortisol (ng/ml)	8.41 ± 2.36	3.75 ± 1.04	0.96
Glucose (mmol/l)	3.74 ± 0.75	5.30 ± 2.69	0.96
Lactate (mmol/l)	0.94 ± 0.12	2.69 ± 0.38	0.98
Haematocrit (%)	30.54± 1.67	0.54 ± 0.12	0.47†

Table 3.1 Estimated basal levels (± S.E.M.)and estimated recovery times (± S.E.M.) at pasture for cortisol, glucose, lactate and haematocrit derived from fitting exponential functions to individual data (Days 1 and 2 combined). Goodness of fit is shown by r^2 values. * The mean asymptotic value for the individual exponential curves. ** The mean time at which the fitted curve attained a value within 10% of the asymptotic value. † Indicates a poor fit to the exponential function hence estimated values may be unreliable.

Data on haematocrit, and plasma lactate and glucose levels did not differ significantly between days 1 and 2, therefore the data for both days were combined. The mean values for haematocrit, lactate and glucose from samples taken manualy were not significantly different from the first sample taken remotely using the dracpac. Levels declined significantly after release from restraint to estimated basal levels of 30.5 % for haematocrit, 0.94 mmol/l for lactate, and 3.74 mmol/l for glucose (Table 1). The times taken to achieve the estimated basal values were 0.5 h for haematocrit, 2.7 h for lactate, and 5.3 h for glucose.

3.5 DISCUSSION

During this study the Dracpac remote blood sampler functioned reliably and resulted in a high percentage return of expected samples. Non-patency of catheters was the reason for reducing animal numbers during the study. This problem has since been rectified through the use of an improved method for fixing catheter lines in place.

We were concerned that the storage of whole blood on the animal for several hours could have affected the levels of some of the important stress indices in the samples. The results of Experiment 1 revealed that levels of cortisol, glucose and haematocrit were unaffected by storage periods of up to 12 h. Lactate levels, however, showed a significant increase over the storage period, though the actual percentage increase was relatively small. These findings are similar to published results with other species (Reimers *et al.* 1983, 1991, Wittwer *et al.* 1986). Hence, the levels of these stress indices in blood collected and stored on the animal would be similar to those in samples collected and processed immediately.

The elevated levels of the four parameters measured in the first samples in Experiment 2 suggests that the procedure for attaching the blood sampler which involved yarding, drafting, and restraint is stressful. Similar high values were reported by Seal *et al.* (1972) for manually restrained white tailed deer (*Odocoileus virginianus*), and by Matthews *et al.* (1990) and Matthews & Cook (1991) in red deer stags restrained in a pneumatic deer crush.

Soon after release to pasture the levels of each of the parameters began to decline. The estimate of the time taken to reach basal values varied from 0.5 h for haematocrit to 5.3 h for glucose. This indicates that a substantial period of time must be allowed to elapse following fitting of the sampler, and before the effects of subsequent stressful events can be assessed.

The estimated mean basal concentration of cortisol was 8.4 ng/ml. This value is similar to that reported for undisturbed red deer stags shot in the field (5.7 ± 3.7

ng/ml, Smith & Dobson 1990) and tame Eld's deer (*Cervus eldi thamin*) sampled by remote catheter (range 5.5 - 14.5 ng/ml, Monfort *et al.* 1993). Similarly, the predicted basal values for lactate, glucose and haematocrit are close to those reported for other unstressed ruminants (Hattingh *et al.* 1988). It would seem that the levels of stress parameters measured in this study are approaching those found in unstressed deer. Thus, it appears that red deer adapt readily to the Dracpac remote blood sampling system and that there is little or no stress associated with its use.

It is envisaged that the Dracpac remote sampler will find application in studies defining variations in a wide range of physiological, haematological and biochemical parameters.

CHAPTER 4 PLASMA CORTISOL RESPONSES TO REMOTE ADRENOCORTICOTROPIC HORMONE (ACTH) INFUSION IN FREE RANGING RED DEER (*CERVUS ELAPHUS*).

4.1 ABSTRACT

A remote infusion and blood collection device (DracPac) was used on free ranging red deer stags to deliver *i.v.* a range of $ACTH_{1-24}$ doses and collect blood for cortisol determination. In Experiment 1, conducted in September, saline and 1, 4 and 16 IU ACTH/100 kg bodyweight were infused (n = 5 to 7 per treatment). In Experiment 2 (November), 16 and 64 IU ACTH/100 kg were infused (n = 5 per treatment); and the response of animals to restraint was also assessed (n = 6).

Pre-infusion concentrations of plasma cortisol were low (mean 6.5 ± 1.0 ng/ml), and unaffected by infusion of saline. ACTH significantly elevated plasma cortisol concentrations, with mean peak concentrations occurring 20 to 40 min post-infusion. Duration of the response was dose dependent, ranging from 80 to 160 min. With increasing doses of ACTH maximal peak heights plateaued at approximately 40 and 60 ng/ml in September and November respectively, whereas the area under the curves tended to increase. The minimum dose of ACTH which resulted in a maximal peak cortisol response was 4 IU. The maximum peak height of the cortisol response to 16 IU tended to be higher

($p<0.10$) in November compared to September, suggesting that adrenal responsiveness may change over this period. The adrenal response to a 16 IU ACTH challenge in Experiment 2 resulted in similar plasma cortisol concentrations to an acute stress event (restraint).

Four IU/100kg ACTH i.v. is recommended as an appropriate dose for ACTH infusion studies in red deer stags. When used in conjunction with the DracPac technique it becomes a stress-free, repeatable procedure for assessing aspects of adrenal cortex physiology in free ranging deer.

4.2 INTRODUCTION

A number of farm animal husbandry practices result in stress and can potentially affect the welfare of animals. When evaluating the welfare impacts of these practices or when comparing alternative procedures, a quantifiable index of the degree of stress experienced by the animal is required. Adrenal cortex function is widely used as such an index (Barnet & Hemsworth 1990), either by monitoring changes in plasma cortisol concentrations during a stressful procedure (e.g. Seal *et al.* 1983, van Mourik & Stelmasiak 1984, Matthews & Cook 1991, Ingram *et al.* 1994) or by assessing the responsiveness of the adrenal cortex to exogenous adrenocorticotropic hormone infusion (ACTH challenge) (e.g. Ladewig & Schmidt 1989, Goddard *et al.* 1994). A number of studies have shown that conditions of chronic stress can alter the responsiveness of the hypothalamo-pituitary-adrenal (HPA) axis to acute stress or ACTH challenge. For example, crowding, social disruption, and competition for free stalls resulted in an increased cortisol response to exogenous ACTH (Friend *et al.* 1977, 1979).

The adrenocortical responsiveness to ACTH has been investigated in a number of deer species, including rusa (van Mourik & Stelmasiak 1984), white tailed (Smith & Bubenik 1990, Bubenik 1991), axis (Bubenik *et al.* 1991), fallow (Asher *et al.* 1989, Bubenik & Bartos 1993) and red (Goddard *et al.* 1994, Jopson *et al.* 1990, Bubenik & Bartos 1993, Hanlon *et al.* 1994, Suttie *et al.* 1995). In all of these studies adrenal responsiveness to ACTH challenge was determined in either

repeatedly physically restrained (Goddard *et al.* 1994, Asher *et al.* 1989, Jopson *et al.* 1990, Hanlon *et al.* 1994), or sedated (Smith & Bubenik 1990, Bubenik & Bartos 1993, Suttie *et al.* 1995) animals. However, the effects of endogenous ACTH, released as part of the HPA axis response to handling and restraint stress, could be a major confounding variable in using such restraint techniques (Suttie *et al.* 1995). Even when using sedated animals, differing levels of sedation over time and between animals makes interpretation of the adrenal response to ACTH or stressors difficult (Monfort *et al.* 1993).

In order to overcome these confounding effects and to make the results from such studies more applicable to extensively farmed deer, a remote infusion and blood sampling system (Ingram *et al.* 1994) for use in free-ranging animals was developed and used to assess adrenocortical responses to bolus ACTH infusion.

Typically, adrenocortical responsiveness in red deer has been studied following administration of large pharmacological doses of ACTH (10-75 IU), often by intramuscular injection (Bubenik & Bartos 1993, Suttie *et al.* 1995), which result in prolonged maximal elevations in plasma cortisol (Bubenik & Bartos 1993). These responses are uncharacteristic of normal HPA axis function and while reflecting the ability of the adrenal to respond to unusual pharmacological challenges, may not adequately characterise the adrenal response to normal stressors encountered by the animal (Broide *et al.* 1995). Evidence from studies on humans shows that ACTH challenge tests using low doses of $ACTH_{1-24}$ (0.5-1 μg / 1.73 m^2) are more sensitive than pharmacological dosages at detecting subtle changes in adrenal function (Dickstein *et al.* 1991, Broide *et al.* 1995).

Since there is no information on adrenocortical responsiveness to low doses of ACTH in undisturbed free ranging red deer, a range of ACTH doses were investigated in Experiment 1 to find a suitable ACTH dose that would consistently produce maximal adrenal stimulation for a period similar to that observed during an acute stressor (Carragher *et al.* 1997).

In a second experiment, some of the same animals were given a pharmacological dose of ACTH to confirm that maximal adrenal output was being achieved in

Experiment 1. In addition, the plasma cortisol concentrations following ACTH infusion were compared with those following a common handling procedure (yarding and restraint); a procedure known to evoke a strong HPA axis response (Carragher *et al.* 1997).

4.3 MATERIALS AND METHODS

4.3.1 Experiment 1 - September

4.3.1.1 Animals

Twelve stags (21 months of age; 75-99 kg) out of a group of 17 male red deer were used. They were kept in 0.25 - 0.50 hectare paddocks with *ad libitum* access to pasture (ryegrass-white clover) and water at the Ruakura Agricultural Centre, Hamilton, New Zealand (37°46'S, 175°20'E).

4.3.1.2 Procedure and remote blood sampling

The experiment took place over three successive weeks. One subgroup of six animals was used in weeks 1 and 3 and a second subgroup in week 2 (Table 1). Each week the animals were used for three days (0,1,2). On Day 0 the six experimental animals were sedated with an i.m. injection of 1 mg kg^{-1} xylazine hydrochloride (Rompun, Bayer, Auckland, N.Z.) and a double lumen catheter was inserted into the jugular vein (Ladewig & Stribrny 1988). The opposing jugular was also catheterised with a single lumen catheter (Cavafix Certo 16G x 32 cm, Braun, Germany) for infusion of ACTH. At the same time all cannulated animals were fitted with a canvas backpack. Sedation was reversed with an i.v. injection of 0.25 mg kg^{-1} yohimbine hydrochloride (Revercyl, Aspiring Animal Services Ltd., Wanaka, N.Z.) and the animals were reunited with the remaining eleven animals and returned as a group to the experimental paddock (0.25 hectare).

The following day (Day 1) the 6 catheterised animals were penned and fitted with a battery powered remote blood sampling and infusion device (DracPac, Engineering Development Group and ABWRC, Ruakura Agricultural Centre, Hamilton, N.Z.).

The external dimensions of the device were 150 x 110 x 60 mm and it weighed 1.3 kg (Figure 4.1)

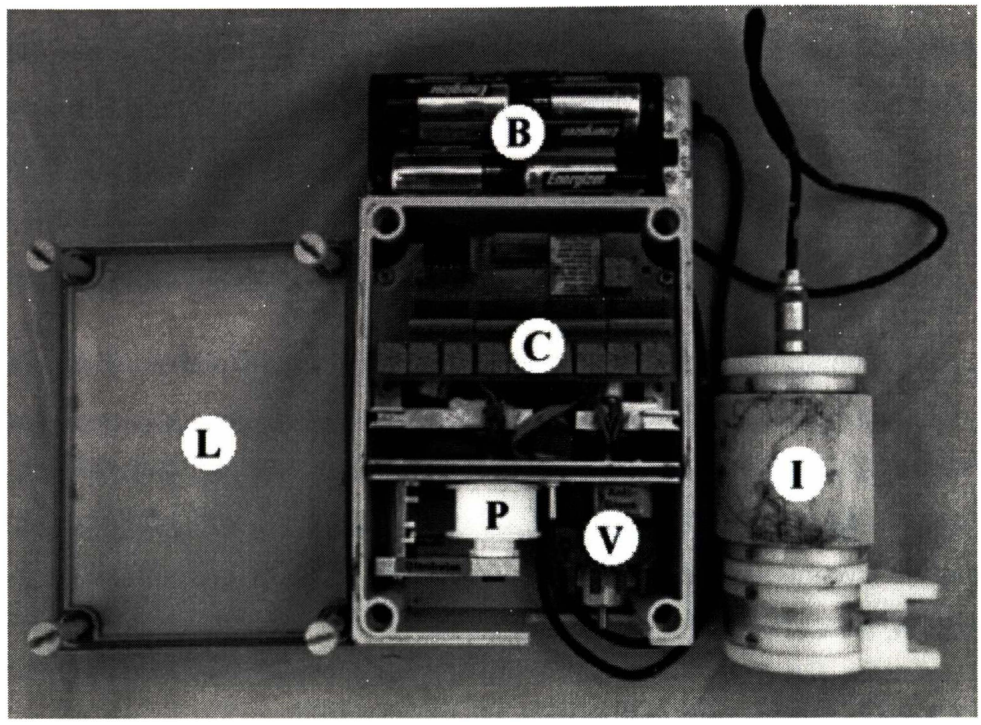


Figure 4.1 Photograph of the DracPac remote blood sampling device with the lid (L) removed showing the battery compartment (B), microprocessor and control unit (C), peristaltic pump (P), 12-position rotary switching valve (V) and the ancillary infusion pump (I) used for infusing ACTH.

The DracPac device consists of two small peristaltic pumps (main and ancillary), a 12-position rotary switching valve (Mayes *et al.* 1988) and a programmable microprocessor unit which controls the start time, duration and rate of sample collection. The main peristaltic pump delivers heparinised saline (5000 IU/ml) down one side of the double lumen catheter, to the tip where it mixes with jugular blood being continuously drawn up the second lumen by the action of the same pump. The heparinised blood then passes through the rotary switching valve and is collected into 1 of 12 separate PVC bags. The samples were stored on ice in an insulated pouch within the backpack until removal and processing.

The DracPac was programmed to pump blood to a waste bag until 14:00 h (3.5 hr after release from the yarding facility) to allow cortisol values to return to baseline after handling (Ingram *et al.* 1994). The pump and valve were programmed to collect blood continuously at a rate of 24 ml per hour for 11 samples, each of 20

minutes duration. Following collection of the last blood sample at pasture, the experimental animals were returned to the yards and the blood sampler and samples removed from the backpack. The procedure of fitting the DracPac and obtaining the samples was repeated the following day (Day 2) after which all equipment was removed from the animals.

4.3.1.3 ACTH infusion

The ACTH doses (treatments) were administered on Days 1 and 2. At the start of the second blood sample (14:20 hr), the ancillary peristaltic pump infused 2 ml physiological saline containing 0, 1, 4 or 16 IU/100 kg of $ACTH_{1-24}$ (Synacthen, Ciba N.Z., Auckland, N.Z.) followed by 5 ml of physiological saline via the single lumen catheter. Infusion of ACTH and flushing the catheter with saline took approximately 5 minutes. A dye marker in the infusion line was used to determine that the ACTH had been administered successfully. Treatments were allocated on a balanced incomplete block design controlling for order and day effect, with each animal receiving 2 of the 4 ACTH doses during any one week (Table 4.1).

4.3.2 Experiment 2 - November

4.3.2.1 Animals

Six of the deer from Experiment 1 were used for one week in November 1994 (24 months of age; 88-110 kg body weight). The animals were maintained under the conditions as described for Experiment 1.

4.3.2.2 Procedure and remote blood sampling

On Days 0, 1 and 2 the procedures were similar to those in Experiment 1 except that only two ACTH doses (16 and 64 IU/100 kg) were used. Doses of ACTH were allocated according to a Latin square design (see Table 1).

On Day 3, the animals were yarded and each in turn was physically restrained in a pneumatic deer restraint device (Heenan Crush, Christchurch, NZ) for 2 minutes. A blood sample was collected by venipuncture after 2 minutes of restraint. Following

release the animals were held in a group of six in an indoor pen. Thirty minutes later the animals were again restrained for 2 minutes and a second blood sample taken.

	Experiment 1						Experiment 2	
Week	1		2		3		1	
Date	7/9	8/9	14/9	15/9	28/9	29/9	9/11	10/11
Stag #								
201	0*	4*			0	1		
203	16	0			4	16		
210	16*	1			16*	1*		
211	1*	0*			1*	0		
212	4	16			4	0*		
216	1	4			0	16		
204			16	4			64	16
205			1	16			16	64*
207			0*	16			16	64
209			4	1			64	16*
214			0	1			16	64
217			4	0*			64	16

Table 4.1 Dose of ACTH allocated to individual animals in Experiments 1 and 2. *denotes either unsuccessful administration of ACTH or collection of blood samples.

4.3.3 Blood sample analyses

All samples were centrifuged after removal from the DracPac and the plasma stored at -20°C until assayed for cortisol. The interval between blood sample collection and processing did not exceed 7 hours. A previous study (Ingram *et al.* 1994) demonstrated that cortisol concentrations in heparinised whole blood did not significantly change when left for up to 12 hours. Plasma cortisol concentrations were determined in duplicate, after extraction in ethyl acetate, by an ^{125}I radioimmunoassay method with polyethylene glycol separation (Ingram *et al.* 1994). A standard curve, using charcoal stripped deer plasma, was also extracted and used to calculate the concentration of cortisol in individual plasma samples. The cross-reactivity of the antiserum with 11 deoxycortisol is 5.7% and cortisone 1.2%. The inter and intra-assay co-efficient of variations for spiked deer plasma controls of known low (5 ng/ml), medium (20 ng/ml) and high (50 ng/ml)

cortisol concentrations were 15.6 % and 11.7 % (low), 10.4 % and 5.8 % (medium), 10.3 % and 8.9 % (high), respectively. Assay sensitivity was 0.48 ng/ml.

4.3.4 Statistical analyses

Peak height (the maximum concentration observed after ACTH infusion), was calculated for individual animals. Area under the curve, (as each continuously collected sample is an integrated value for that sampling period) the sum of the first eight values (ie. 160 minutes) post-infusion minus the pre-infusion cortisol concentration, were calculated. One hundred and sixty minutes post-infusion was chosen as the cut off point as none of the mean response curves were significantly elevated above baseline values beyond this point.

Data were analysed using the residual maximum likelihood procedure (REML) in the Genstat 5 (v. 3.1) statistical package (Lawes Agricultural Trust 1997). Individual animal effect was included as a factor in the model when it was found to be significant ($p<0.05$). The REML procedure allows the significance of effects to be estimated using likelihood ratio tests. The two experiments were analysed separately. However, a third analysis was carried out comparing the responses to 16 IU ACTH in September and November to determine if time of year affected the cortisol response. For both Experiments 1 and 2 the duration of the cortisol concentration elevation above baseline within a treatment was determined by Student's t tests. Differences were considered to be significant if $p<0.05$. All values are shown as means and least significant differences (Figs. 1 & 2) or s.e.m. (in text).

4.4 RESULTS

4.4.1 Experiments 1 and 2

The changes in plasma cortisol concentrations following ACTH administration in Experiments 1 and 2 are presented in Figures 4.2 and 4.3, respectively. Pre-infusion baseline concentrations were not significantly different between treatment groups or months (means, 4.0 ± 2.1, 4.7 ± 2.0, 5.1 ± 2.0, 9.6 ± 1.8 ng/ml

for 0, 1, 4 and 16 IU ACTH, respectively, in September and 5.1 ± 3.9 and 9.6 ± 3.9 ng/ml for 16 and 64 IU ACTH in November.

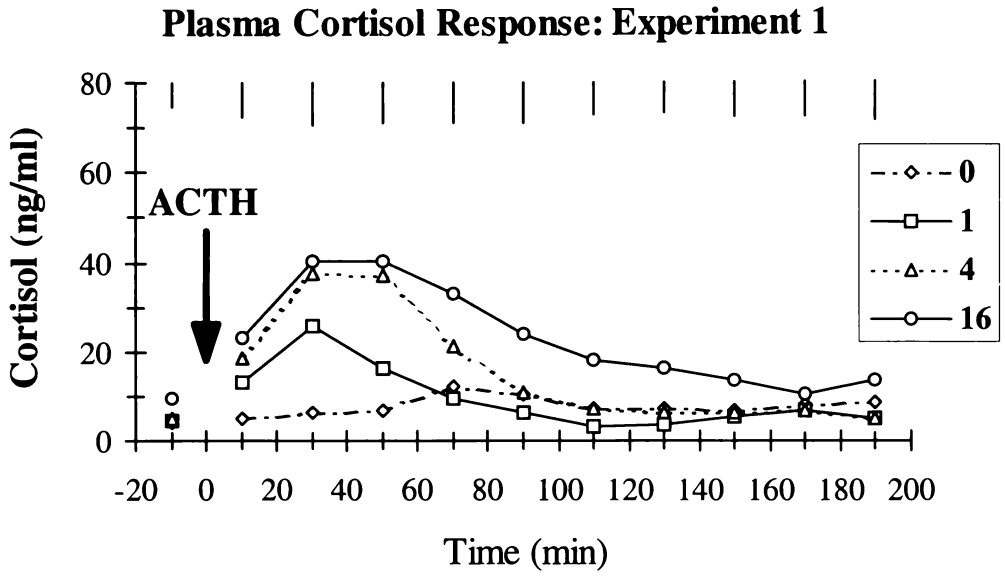


Figure 4.2 Mean plasma cortisol concentrations for red deer stags in September (Experiment 1), before and after bolus infusion (i.v.) (at time zero) of either saline (0) or 1,4 and 16 IU\100kg of synthetic ACTH (Synacthen) at time 0. Each point is the mean cortisol level measured in an integrated blood sample taken over a 20 minute period. Vertical bars denote least significant difference.

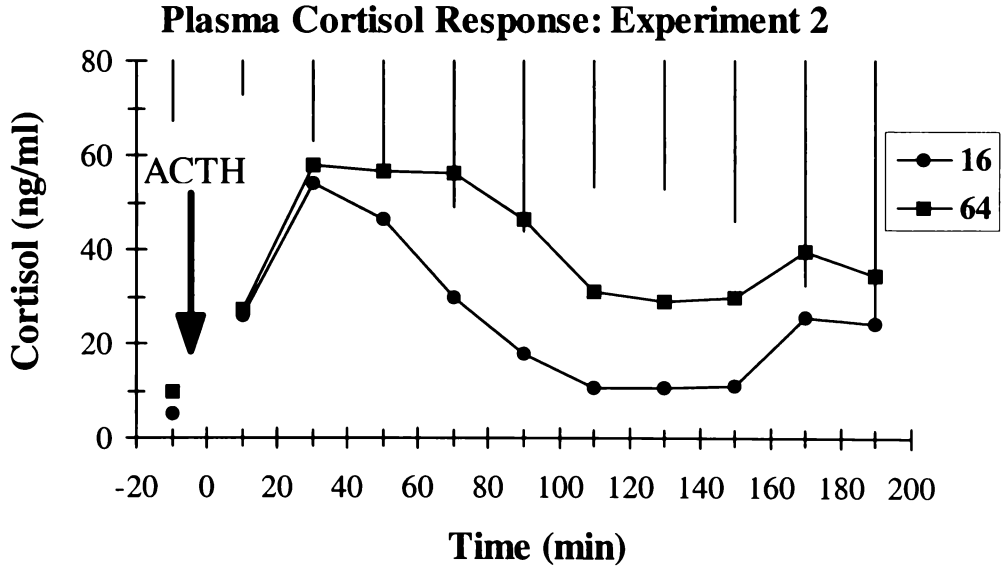


Figure 4.3 Mean plasma cortisol concentrations for red deer stags in November (Experiment 2), before and after bolus infusion (i.v.) (at time zero) of either 16 or 64 IU\100kg of synthetic ACTH (Synacthen) at time 0. Each point is the mean cortisol level measured in an integrated blood sample taken over a 20 minute period. Vertical bars denote least significant difference.

There was no significant plasma cortisol response attributable to the infusion of saline alone. However, plasma cortisol concentrations following administration of ACTH in Experiment 1 were significantly higher than the pre-infusion baselines for 80, 120 and 160 min, for the 1, 4 and 16 IU doses, respectively. In Experiment 2, the cortisol response to 16 IU ACTH was significantly higher than the pre-infusion baseline for 100 min post-infusion; whereas 64 IU gave a response which was significantly different from the baseline value for only 80 min. There was a large variation in the duration of the response to the 64 IU dose between individuals, with plasma cortisol concentrations having decreased to baseline in only two of the five animals by the end of the sampling period.

Maximum cortisol peak heights to the 4 and 16 IU infusions in Experiment 1 (September) were significantly higher than that to 1 IU, and all three were higher than the peak concentration measured after infusion of saline (saline, 14.3 ± 3.6; 1 IU, 26.3 ± 3.3; 4 IU, 38.3 ± 3.3; 16 IU, 43.2 ± 3.4 ng/ml). The 4 and 16 IU maxima were not significantly different from each other (54.9 ± 6.7 and 65.8 ± 6.7 ng/ml respectivly). In Experiment 2 (November) the maximum peak cortisol concentrations following the 16 and 64 IU doses were not significantly different. The maximum response to 16 IU tended to be higher in November (54.9 ± 4.0 ng/ml) than September (43.2 ± 3.4 ng/ml) ($p<0.10$). Further, the three animals that received 16 IU in both September and November had significantly higher peak concentrations ($p<0.05$) in November, with an average increase of 16.5 ± 2.6 ng/ml or 36.9 ± 3.6 % over the September peak values.

Mean maximum plasma cortisol concentrations were reached by the 20 to 40 minute post-infusion blood sample, irrespective of dose or month. A range in time to peak was observed in the individual response curves with a tendency for the higher ACTH dose peaks to occur after the 20 to 40 minute sample.

The magnitudes of the cortisol responses to ACTH dose, as measured by the area under the curve, were saline 29.9 ± 15.2; 1 IU, 45.6 ± 13.9; 4 IU, 104.6 ± 13.9; 16 IU, 131.9 ± 12.9 ng/ml/160min for September and 165.5 ± 73.7 and 256.3 ± 82.4 ng/ml/160min for 16 and 64 IU respectively in November. For the September data there was overall, a highly significant effect of ACTH dose ($p< 0.001$) on

area under the curve although the response to 1 IU was not significantly different from the saline control. Both of the higher ACTH doses in September resulted in areas which were significantly different from saline and 1 IU, but not from each other. There was no significant difference in area under the cortisol response curves for the 16 and 64 IU doses in November, nor between the 16 IU responses in September and November.

The mean cortisol concentrations measured in plasma taken from animals whilst they were being physically restrained in a pneumatic crush on Day 3 of Experiment 2 were 43.1 ± 1.6 for the first sample and 49.1 ± 7.0 ng/ml 30 min. later. The 30 min. value was not significantly different from the 20 - 40 min post-infusion cortisol concentrations of either the 16 or 64 IU treated animals (54.1 and 58.0 ng/ml, respectively).

4.5 DISCUSSION

This study has for the first time determined the plasma cortisol response of undisturbed, free ranging red deer stags to a range of ACTH doses delivered and monitored by a remote blood sampling device (Ingram *et al.* 1994).

One of the main features of the results was the low plasma cortisol concentrations in the pre-infusion baseline samples (overall mean 6.5 ± 1.0 ng/ml) and the low concentrations during and after infusion of saline (range of means 4 - 13.6 ng/ml). These values are considerably lower than those reported as baselines in all other studies on deer where animals have been physically or chemically restrained prior to blood sampling (6 - 27 ng/ml, Jopson *et al.* 1990; 18 - 27 ng/ml, Goddard *et al.* 1994; 17 - 55 ng/ml, Bubenik & Bartos 1993) but are in agreement with values from previous studies carried out using the DracPac remote sampling device (8.4 ± 2.4 ng/ml, Ingram *et al.* 1994) and for undisturbed red deer stags shot dead at pasture immediately prior to sampling (5.7 ± 3.7 ng/ml, Smith & Dobson 1990). This indicates that in previous studies, restraint artefacts have been a potential confounding factor in the interpretation of ACTH challenge results.

The animals responded to the remote bolus (*i.v.*) infusion of $ACTH_{1-24}$ (Synacthen, Ciba-Geigy) in a similar dose dependent manner to that reported previously for red deer (Goddard *et al.* 1994) and other domesticated species (sheep, Fulkerson & Jamieson 1982; cattle, Verkerk *et al.* 1994). Plasma cortisol concentrations increased immediately after ACTH infusion and peaked during the 20 to 40 minute sample period (Goddard *et al.* 1994).

However, only relatively low doses of ACTH were required to achieve maximum cortisol output, as measured by peak height. Thus, the two higher doses (4 and 16 IU) used in Experiment 1 produced similar peak heights, and the peak heights following infusion of 16 & 64 IU in Experiment 2 were not significantly different from each other. This plateau effect has been observed in other ACTH challenge studies in deer (Goddard *et al.* 1994) and cattle (Verkerk *et al.* 1994). The maximum peak height observed after administration of 1 IU in Experiment 1 was significantly lower than that obtained for 4 and 16 IU. This could be due either to the 1 IU ACTH dose been insufficient to achieve maximum adrenal output, or that the blood sampling frequency used (continuous 20 minute samples) may not accurately quantify short duration peaks. This limitation may lead to underestimation of peak heights for low ACTH doses or less severe acute stressors. This could be circumvented by increasing the sampling frequency to improve resolution of peaks.

The maximal cortisol elevations observed after administration of a relatively low dose of ACTH (4 IU/100kg) in Experiment 1, highlights the sensitivity of the red deer adrenal cortex to exogenous ACTH. A dose of 4 IU/100kg is below the range of values reported to elicit maximal responses in deer (rusa, 6 IU/100kg (van Mourik & Stelmasiak 1984); red, 12.9 IU/100kg (Godard *et al.* 1994)) and sheep (28 IU/100kg (Fulkerson & Jamieson 1982)), and is at the lower end of the range in published studies for other domesticated species (cattle, 2.1 IU/100kg (Verkerk *et al.* 1994)).

The maximum cortisol concentrations reported for cervids after ACTH challenge varies depending on the species and between studies, with 80 to 160 ng/ml reported for fallow deer (Asher *et al.* 1989, Bubenik & Bartos 1993), 120 ng/ml

for rusa deer (van Mourik & Stelmasiak 1984) and over 200 ng/ml for white tailed deer (Smith & Bubenik 1990). The peak cortisol concentrations obtained in this study fall within the range reported for red deer following ACTH challenge (females: 40 to 90 ng/ml (Goddard *et al.* 1994, Jopson *et al.* 1990); males: 100 ng/ml, (Bubenik & Bartos 1993)) and following routine handling procedures (males: 25 to 70 ng/ml (Matthews & Cook 1991, Ingram *et al.* 1994, Carragher *et al.* 1997, Matthews *et al.* 1994)).

The area under the cortisol response curve increased with increasing ACTH dose, which is consistent with observations in other species (cattle (Verkerk *et al.* 1997)). This reflects the prolonged period of elevation of cortisol concentrations at higher doses as reported in other published studies with deer (van Mourik &Stelmasiak 1984, Goddard *et al.* 1994) and cattle (Verkerk *et al.* 1994), rather than changes in peak height. However, variation between individuals (particularly with 64 IU) and the relatively small number of animals tested in an incomplete Latin square experimental design, has tended to obscure some effects. For example, the highly variable response to 64 IU may reflect differences in the ability of individual deer to maintain a prolonged response to high "pharmacological" doses of ACTH or differences in individual clearance rates of synthetic ACTH. However, large numbers of animals would be required to demonstrate this.

In the present study a difference in maximum peak height between September and November was observed. The tendency for a higher peak cortisol concentration obtained following infusion of 16 IU ACTH in November compared with September may indicate a change in adrenal responsiveness. A recent study of adrenal responsiveness in red deer stags (Suttie *et al.* 1995) has also shown a greater plasma cortisol response to ACTH challenge during late velvet growth (November) compared with times during antler casting and early velvet growth (September). Another recent study (Verkerk & Macmillan 1997) has also shown that reproductive status can alter adrenal responsiveness to ACTH in cattle, with bulls having a lower response than castrated animals of the same age. Red deer stags exhibit marked annual changes in behaviour and physiology that are associated with reproduction; changing from being essentially functional castrates

during late spring and early summer to having a fully functional reproductive axis during the breeding season (autumn and early winter) (Suttie *et al.* 1992). Mean concentrations of testosterone have been reported to decline through September (1 ng/ml) to reach a nadir during October to November of 0.2 ng/ml (Suttie *et al.* 1992). Other possible explanations for the higher response observed in November include physiological and behavioural changes associated with the growth of new velvet antler (evident in November), or changes in environmental factors such as improving feed supply and/or increasing day length and ambient temperature.

Different characteristics of the cortisol response to ACTH challenge (maximum peak height, area under the curve) may have different diagnostic functions. Recent studies on humans have shown that the peak plasma cortisol concentration obtained following ACTH challenge is the best criterion for measuring changes in adrenal function (Dickstein *et al.* 1991). Thus, maximum peak cortisol concentrations may be useful for identifying changes in adrenal responsiveness to environmental influences or management practices in deer. Area under the cortisol response curve which did not plateau with increasing ACTH dose (at least for the range of ACTH doses used in this study), may be more useful as an indicator of the amount of ACTH either infused or released following stress (Friend *et al.* 1979). This suggests that it would be important to obtain a detailed time series profile of the adrenal response to challenge for valid interpretation of results. The technique of remote infusion and blood sampling using the DracPac is ideal for this function.

To determine if the doses of ACTH tested were at physiologically relevant levels, the animals in Experiment 2 were subjected to a known stressful procedure, restraint in a pneumatic drop floor crush (Carragher *et al.* 1997, Matthews *et al.* 1994). This procedure resulted in peak plasma cortisol concentrations similar in magnitude to those found 20 - 40 min post-infusion for the two doses (16 and 64 IU/100kg) used in Experiment 2. Furthermore, the peak height and duration of the cortisol responses reported for acute handling procedures (Ingram *et al.* 1994, Carragher *et al.* 1997, Matthews *et al.* 1994) were similar to the responses to 4 and 16 IU used in the present study. These data suggest that the lower doses of

ACTH (1-16 IU/100kg) employed in the present study were not supraphysiological.

The results of this study will assist in identifying suitable ACTH doses for further investigation of adrenal cortex function. Studies on humans show that challenge tests using low doses of ACTH are more sensitive for detecting subtle changes in adrenal function (Broide *et al.* 1995, Dickstein *et al.* 1991). In the present study, 4 IU was the minimum ACTH dose which resulted in maximal peak cortisol concentrations. In addition, 4 IU produced a duration of cortisol elevation which was similar to that observed after an acute stressor (Ingram *et al.* 1994, Carragher *et al.*1997, Matthews *et al.* 1994). Therefore, 4 IU/100kg could be the ideal dose for evaluating the effects of prior stressors on adrenocortical function.

In summary, the present study demonstrates the usefulness of the DracPac device to remotely deliver and monitor ACTH challenges to free ranging animals without the confounding effects of repeated handling and restraint stress. The results highlight the sensitivity of the adrenal cortex of free ranging red deer to low doses of ACTH. Future studies will utilise the remote ACTH challenge technique to evaluate changes in adrenal function of free ranging red deer that may be associated with seasonal, environmental and management factors.

CHAPTER 5 ULTRADIAN, CIRCADIAN AND SEASONAL RHYTHMS IN CORTISOL SECRETION AND ADRENAL RESPONSIVENESS TO ACTH AND YARDING IN UNRESTRAINED RED DEER (*CERVUS ELAPHUS*) STAGS.

5.1 ABSTRACT

Seasonal changes in the activity and responsiveness of the adrenal gland in red deer (*Cervus elaphus*) stags were quantified by measuring 24 h endogenous cortisol secretory profiles and plasma cortisol responses to either administration of exogenous ACTH or a standardised stressor during November (period of velvet growth), February (pre-rut), April (mid-rut) and July (post-rut) (southern hemisphere) using a remote blood sampling device (DracPac).

Ultradian rhythms in the concentration of plasma cortisol were observed resulting from the episodic secretion of cortisol from the adrenal cortex at a mean rate of 0.8 pulses/h. Circadian rhythms in plasma cortisol concentrations were also found in 11 out of the 20 complete 24 h profiles (mean amplitude, 3.8 ± 1.4 ng/ml).

Seasonal rhythms in mean 24 h plasma cortisol concentrations and cortisol pulse parameters were also observed. Mean 24 h plasma cortisol concentrations were higher in November (12.5 ± 1.0 ng/ml) than February (6.3 ± 1.0 ng/ml), April (4.0 ± 1.0 ng/ml) or July (4.2 ± 1.0 ng/ml). Cortisol pulse height, nadir and amplitude were all significantly higher in November than at other times of year ($p < 0.01$).

Peak cortisol concentrations following infusion of $ACTH_{1-24}$ (0.04 IU kg^{-1}) were higher ($p < 0.05$) in November (55.8 ± 2.7 ng/ml) and lower ($p < 0.001$) in April (33.7 ± 1.8 ng/ml) than those in February and July (48.7 ± 2.0 ng/ml and 45.4 ± 2.0 ng/ml, respectively). The area under the cortisol response curve was significantly smaller ($p < 0.05$) in April (266.6 ± 15.3 ng/ml/190min) than at other times of year (February, 366.1 ± 15.3 ng/ml/190min; July, 340.7 ± 15.3 ng/ml/190min and November, 387.8 ± 21.2 ng/ml/190min).

These data demonstrate that the adrenal gland of the red deer stag exhibits ultradian, circadian and seasonal rhythms in activity, and that its responsiveness to ACTH varies with season. November, a period of reproductive quiescence in the southern hemisphere, with new antler growth and rapid weight gain, is associated with higher mean plasma cortisol concentrations and a greater responsiveness to exogenous ACTH. In contrast, the breeding season is associated with lower adrenal activity and responsiveness.

5.2 INTRODUCTION

Changes in the activity and function of the hypothalamic-pituitary-adrenal (HPA) axis are routinely used to assess welfare in farm animals (Barnett & Hemsworth 1990, Fraser & Broom 1990). Studies in a variety of species including human (Veldhuis *et al.* 1990), cattle (Ladewig & Smidt 1989), sheep (Kennaway *et al.* 1981) and deer (Monfort *et al.* 1993) have shown that the HPA axis exhibits variations over time in basal activity and responsiveness, distinct from that following the imposition of a stressor. This variation can form a consistent rhythm which fluctuates within or about 24 h (ultradian and circadian rhythms,

respectively) or can be of longer duration, with variations occurring on an annual basis (seasonal or circannual rhythms).

Ultradian rhythms in plasma concentrations of glucocorticoids are the result of episodic secretion of glucocorticoid pulses from the adrenal cortex. Such pulsatile secretion is thought to prevent down-regulation of the HPA axis while maintaining it's ability to respond to stress (Monfort *et al.* 1993). In deer, episodic secretion of cortisol, the predominant glucocorticoid (van Mourik *et al.* 1985, Smith & Bubenik 1990), has been investigated in Eld's deer (*Cervus eldi thamin*) (Monfort *et al.* 1993) but there appears to be no information on cortisol secretory dynamics in red deer (*Cervus elaphus*).

Circadian rhythms in plasma glucocorticoid concentrations are well characterised for humans (Weitzman *et al.* 1971) and rodents (Keller-Wood & Dallman 1984), with peak concentrations preceding the activity phase of the daily cycle. This rhythm is entrained by feeding (Saito *et al.* 1989) and activity/sleep wake cycles (Born *et al.* 1997). There is no evidence from studies of deer for a circadian rhythm in HPA axis activity (white-tailed deer (*Odocoileus virginianus*), Bubenik *et al.* 1983; rusa deer (*Cervus timorensis*), van Mourik & Stelmasiak 1984; Eld's deer, Monfort *et al.* 1993). However, in these studies the deer were subjected to either handling or indoor housing, factors known to attenuate circadian rhythms in other species (Irvine & Alexander 1994).

Seasonal changes in activity and responsiveness of the HPA axis have been reported in a number of species including humans (Walker *et al.*1997), primates (Schiml *et al.* 1996), rodents (Boswell *et al.* 1994) and fish (McLeese *et al.* 1994). Evidence for a seasonal rhythm in adrenal activity or responsiveness in deer varies between species and studies, with seasonal changes reported in white-tailed deer (Bubenik *et al.* 1983), reindeer (*Rangifer tarandus*) (Nilssen *et al.* 1985), axis deer (*Axis axis*) (Chapple *et al.* 1991) and red deer (Suttie *et al.* 1995, Cassidy 1996) but not in Eld's deer (Monfort *et al.* 1993) or in another study on axis deer (Bubenik & Brown 1989).

Red deer stags undergo dramatic seasonal changes in physiology and behaviour associated with the breeding season (rut). These include changes in secretion of hormones involved in the reproductive (Lincoln & Kay 1979, Suttie *et al.* 1992) and growth and metabolic axes (Suttie *et al.* 1989). Changes in behaviour at this time include increased aggression (Suttie1985), and a reduced voluntary food intake (Loudon *et al.* 1989) resulting in pronounced weight loss (Kay 1979). It is well documented in other mammalian species that HPA axis activity can be modulated by changes in reproductive function (increased activity and responsiveness in castrates compared with entire males (Bass *et al.* 1982, Verkerk & Macmillan 1997)) and changing metabolic and growth demands (Yanovski *et al.* 1997) as well as by social factors (Lyons *et al.* 1988). This has yet to be demonstrated for red deer.

Changes in responsiveness of the HPA axis as assessed by the adrenal response to physiological (e.g. ACTH infusion, insulin induced hypoglycemia) or psychological (e.g. handling, open field test) challenges are commonly used in assessing the normal functioning of the HPA axis in animal stress research. Seasonal changes in adrenal responsiveness to ACTH challenge have recently been demonstrated in red deer stags (Suttie *et al.* 1995, Cassidy 1996) but there have been no studies of seasonal variation in response to stress or basal activity of the HPA axis.

The aim of the current study was to identify and quantify ultradian, circadian and seasonal rhythms in basal cortisol secretion of red deer stags. In addition, we sought to measure seasonal changes in adrenal and HPA axis responsiveness following challenges with ACTH and a standardised management stressor.

Routine handling of red deer (eg. yarding, drafting and restraint), has been shown to result in activation of the HPA axis (Ingram *et al.* 1994, 1997, Carragher *et al.* 1997). To avoid the confounding effects of handling stress on basal HPA axis activity, a remote blood sampling device (Ingram *et al.* 1994) was used to obtain blood samples from undisturbed animals at pasture.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Twelve 3-year-old red deer stags were maintained at the Ruakura Agricultural Centre, Hamilton, New Zealand (37°46'S, 175°20'E). Two groups of 6 animals, balanced for weight, were formed in July 1995. Each group was kept in a separate 0.25 hectare paddock during the experiment and in 0.25-0.5 hectare paddocks between experiments, with *ad libitum* access to pasture (ryegrass-white clover) and water.

5.3.2 Procedure and remote blood sampling

On four separate occasions throughout the year (1/11/95, 7/2/96, 10/4/96 and 10/7/96), blood samples were collected from the same 3 animals in each group over a 24 h period (24 h profile) and following two types of challenge (ACTH and yarding). Animals from 2 separate groups were used so that data were obtained from 2 replicates, thereby reducing the chances that features unique to one group would influence the results obtained. The experimental procedure was similar for each season, as detailed below.

On Day 1 all animals were weighed and the animals to be blood sampled were sedated with an i.m. injection of 1 mg kg^{-1} xylazine hydrochloride (Rompun, Bayer, Auckland, N.Z.) and a modified double lumen catheter (Cavafix Duo16/18G 32cm, B. Braun, Melsungen, Germany) (see Diagram 1) was inserted into the jugular vein. At the same time all cannulated animals were fitted with a canvas and leather backpack. Sedation was reversed with an i.v. injection of 0.25 mg kg^{-1} yohimbine hydrochloride (Revercyl, Aspiring Animal Services Ltd., Wanaka, N.Z.) and the animals were then reunited with the remaining 3 animals in their group and returned to their respective paddocks.

The following day (Day 2) the 24 h profile blood samples were collected. Both groups were penned between 06:00 and 10:30 h and each of the 6 experimental animals fitted with a battery powered remote blood sampling device (DracPac,

Engineering Development Group, HortResearch and ABWRC, AgResearch, Ruakura Agricultural Centre, Hamilton, N.Z.).

The DracPac device (Figure 5.1,5.2) consists of a pump unit containing two small peristaltic pumps for pumping heparin and drawing blood (length 150 mm, diameter 50 mm, weight 395 g), two 38-position rotary switching valves (length 200 mm, diameter 60 mm, weight 950 g), a 6 volt battery supply (Duracel, MN908) and a control box (length 160 mm, width 80 mm, height 60 mm, weight 580 g) containing a programmable microprocessor which allows programming of the start time, duration and rate of sample collection. The heparin pump delivers heparinised saline (5000 IU/ml) down one side of the double lumen catheter to the tip where it mixes with jugular blood being continuously drawn up the second lumen by the action of the blood pump. Heparin was delivered at a rate of 1.1 ml/h on Days 2-3 and 2.6 ml/h on Day 4. The heparinised blood passed through one or both rotary switching valves and was collected into 1 of 74 separate blood collection tubes (4.5 ml monovette syringe, Sarstedt Ltd., Numbrecht, Germany). The samples were stored on ice pads (Dry Chill, Warragul, Vic., Australia) in an insulated pouch within the backpack. Cortisol concentrations remain stable in heparinised whole blood samples collected from red deer and stored in this fashion for at least 24 h (JR Ingram, unpublished data). Cortisol concentrations are also stable in heparinised whole blood of cattle stored at room temperature for up to 72 h (Reimers *et al.* 1983).

The DracPac was programmed to pump blood to waste until 14:00 h (3.5 h after release from the yarding facility) to allow cortisol concentrations to return to baseline after handling (Ingram *et al.* 1994). The device was programmed to collect blood continuously at a rate of 15 ml per hour for 74 samples, each of 20 minutes duration. Following collection of the last blood sample (14:40 h Day 3), the animals were returned to the yards and the DracPac and blood samples were removed from the backpack.

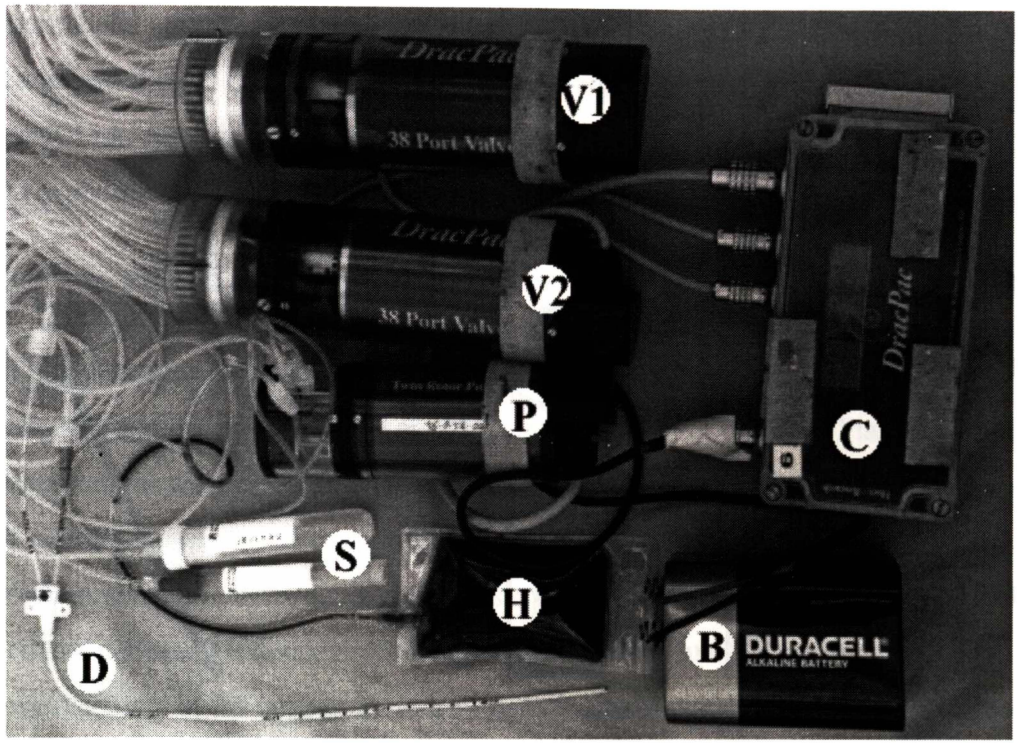


Figure 5.1 Photograph of the latest version of the DracPac remote blood sampling system used for automatic continuous sampling of blood from free ranging animals The device comprises of a microprocessor control box (C); a peristaltic pump unit (P), a plastic bag containing concentrated heparin (H), two 38 port rotary switching valves (V_{1-2}), 74 separate blood collection tubes (S) (4.5 ml monovette syringe, Sarsdetd Ltd., Numbrecht, Germany) a double lumen catheter (D)(Cavafix Duo16/18G 32cm, Braun, Germany) and is powered by a 6 volt alkaline battery (B) (Duracel, MN908).

On Day 4, blood samples were collected before, during and after two types of challenge (ACTH $_{1-24}$ infusion and a yarding stressor). The 6 experimental animals were fitted with the remote blood sampling devices as on Day 2, and returned to their respective paddocks. The DracPacs were programmed to pump blood to waste until 12:30 h (3.5 h after release from the yarding facility), then to collect two 10 min baseline samples (30 ml/h). Immediately following the second baseline sample the DracPac was programmed to infuse 2 ml of physiological saline containing 0.04 IU/kg live weight of ACTH $_{1-24}$; (Synacthen; Ciba NZ, Auckland, NZ) over 5 min into the jugular vein. Fresh blood was then drawn from the animal through the blood lines to a waste tube for 5 min to clear the blood lines of saline. Following the ACTH infusion and flushing of blood lines (total time 10 min) eighteen 10 min blood samples (30 ml/h) were collected over 180 min.

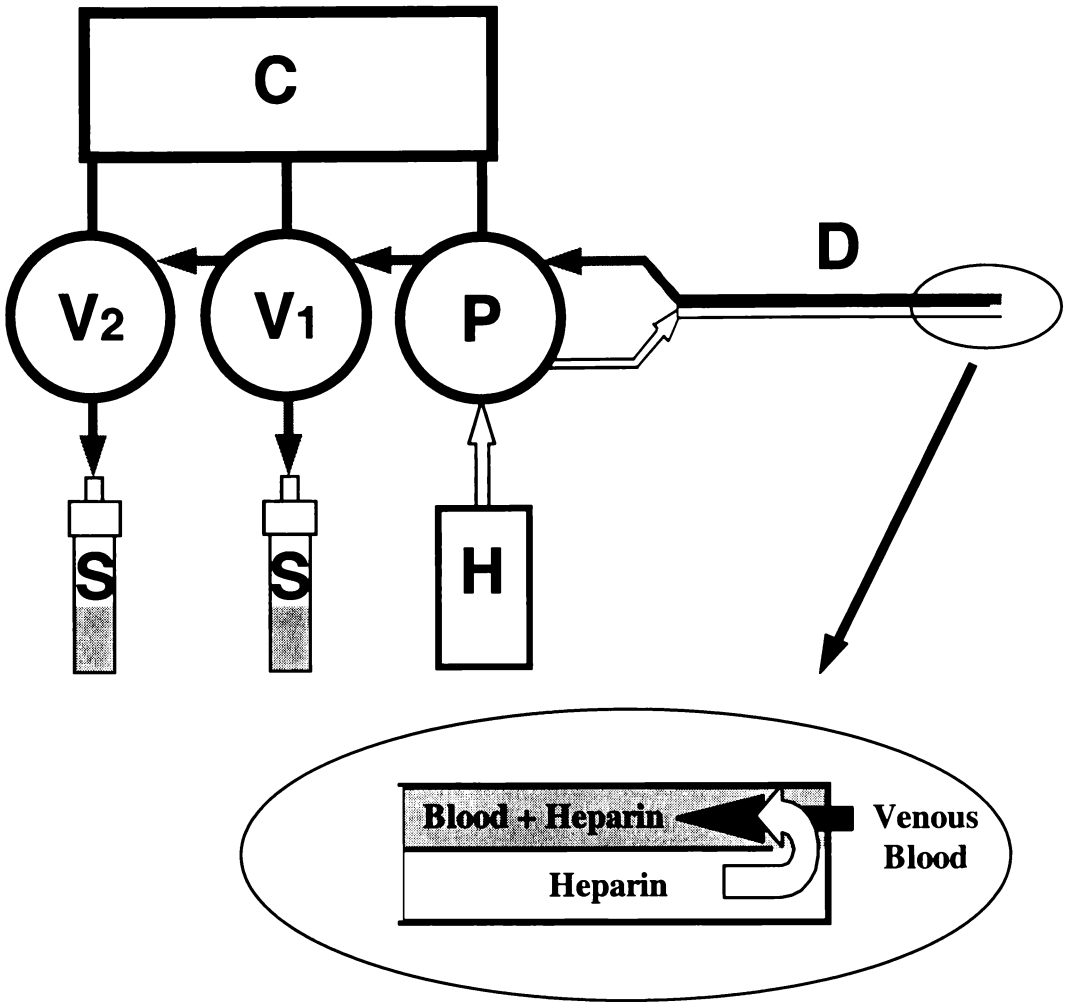


Figure 5.2 Schematic representation of the latest version of the DracPac remote blood sampling system used for automatic continuous sampling of blood from free ranging animals. The device comprises of a microprocessor control box (C); a peristaltic pump unit (P), a plastic bag containing concentrated heparin (H), two 38 port rotary switching valves (V_{1-2}), 74 separate blood collection tubes (S) (4.5 ml monovette syringe, Sarsdetd Ltd., Numbrecht, Germany) and a double lumen catheter (D)(Cavafix Duo16/18G 32cm, Braun, Germany) which is modified by shortening the end of the catheter by ~5cm and removing 1-2 mm of the wall between the two lumina at the tip to facilitate optimal mixing of the outflowing heparin and inflowing blood (see Ladewig & Stribrny, 1988).

Three hours after the ACTH challenge (1600 h) the animals were subjected to the yarding procedure. This comprised bringing the animals in their respective groups of six from their experimental paddocks into the yarding facility and keeping them in an indoor pen (2.4 x 2.4 m) for 10 min before returning them to their paddock (total time from leaving the paddock, yarding and return to the paddock was 30 min). Eight 10 min blood samples (30 ml/h) were collected during yarding and for 50 minutes subsequently at pasture. Following collection of the last blood sample, the

experimental animals were returned to the yards and the catheter, blood samples and all equipment removed. A prophylactic dose of antibiotic (Tripen LA, Ethical Agents Ltd., Auckland, N.Z) was administered i.m. after removal of the catheter. The experimental protocol was approved by the Ruakura Animal Ethics Committee.

5.3.3 Plasma cortisol analyses

All blood samples were centrifuged (1200 g for 15 min) after removal from the backpacks and the plasma stored at -20°C until assayed for cortisol. Plasma cortisol concentrations were determined in duplicate, after extraction in ethyl acetate, by an ^{125}I radioimmunoassay method with polyethylene glycol separation (Ingram *et al.* 1994). A standard curve, using charcoal stripped deer plasma, was constructed and used to determine the concentration of cortisol in individual plasma samples. The cross-reactivity of the antiserum with 11 deoxycortisol was 5.7% and with cortisone was 1.2%. The mean inter and intra-assay co-efficient of variations for spiked deer plasma controls of known low medium and high cortisol concentrations were 13.1 and 13.8% respectively. Assay sensitivity was 0.9 ng/ml.

5.3.4 Statistical analysis

5.3.4.1 Pulse detection

Discrete pulses in cortisol secretion were quantified using cluster analysis (Veldhuis & Johnson, 1986), a statistically based peak detection algorithm. A cortisol pulse was defined as a statistically significant increase in a cluster of cortisol concentrations which preceded a significant decrease. A cluster size of one point for the nadir and one point for a peak was used along with a constant 14% coefficient of variation and a pooled t statistic of 1 to limit the false peak detection rate to approximately 10 % (Veldhuis & Johnson, 1986). The pulse parameters measured for each 24 h profile were pulse frequency, pulse height, pulse amplitude, nadir and peak width.

5.3.4.2 Circadian rhythm detection

Circadian rhythms in plasma cortisol concentrations were detected using a single cosinor model developed for Microsoft Excel (Microsoft Corp., USA) (Bourdon *et*

al. 1995). The amplitudes and times of peak concentrations (acrophase) were obtained using this model in which the data were represented by the best fitting cosine function using the least squares calculation.

5.3.4.3 Statistical analyses

Plasma cortisol concentrations are represented as means ± S.E.M.. Statistical comparisons were performed using analysis of variance (MINITAB for Windows, Release 11.21, Minitab Inc., PA, USA). A blocking structure was used to separate the between and within animal variation. When significant ($p < 0.05$) trends were present, between month comparisons were made using Student's t test.

For the ACTH and yarding cortisol responses, the baseline values were calculated as the average of the 2 samples collected before the imposition of the treatment. Peak heights were defined as the maximum cortisol concentration observed after treatment initiation. Blood sample collection using the DracPac device was by continuous withdrawal over the 10 min sampling period. Therefore, cortisol concentrations for each sample represented an integrated value for that sampling period allowing the area under the cortisol response curve to be calculated by summing the measured concentrations observed after imposition of the treatments (190 min and 70 min for ACTH challenge and yarding stressor, respectively).

5.4 RESULTS

The percentage of samples collected successfully using the remote blood sampling device was 90 % for the 24h sampling (Days 2-3) and 77 % during the challenges. Loss of samples was mainly due to severing of blood collection lines between the catheter and the blood sampler.

Animal live weights increased significantly ($p < 0.001$) from a mean of 116.7 ± 2.3 kg in November 1995 to a peak of 135.3 ± 2.3 kg in February 1996. Mean live weights tended to decline in April (131.8 ± 2.3) and again in July (127.2 ± 2.3 kg), though these differences were not statistically significant ($p > 0.05$).

5.4.1 Twenty four hour cortisol profiles

Representative profiles of cortisol concentration over 24 h for 2 stags (one from each replicate group) for each month are presented in Figure 5.3. Cortisol profiles were characterised by a pulsatile secretory pattern and described by the following parameters; pulse frequency, mean pulse height, mean pulse amplitude, mean nadir and mean pulse width. The mean values for the six experimental animals for each of the four months are shown in Table 5.1. The frequency of cortisol pulses did not differ significantly between November, February and July, though pulse frequencies obtained in April were significantly lower ($p < 0.01$) than those in November and July.

On average, cortisol pulses occurred once every 72 min, a frequency of 0.8 peaks/h. The height, amplitude and nadir of the cortisol pulses were significantly higher ($p < 0.001$) in November than at other times of the year. Cortisol pulse duration was significantly longer ($p < 0.05$) in April compared with November and July. Pulses in February were of intermediate duration and not significantly different. Individual mean 24 h plasma cortisol concentrations ranged from 1.9 to 22.5 ng/ml over seasons and were significantly higher ($p < 0.001$) in November than at other times of year.

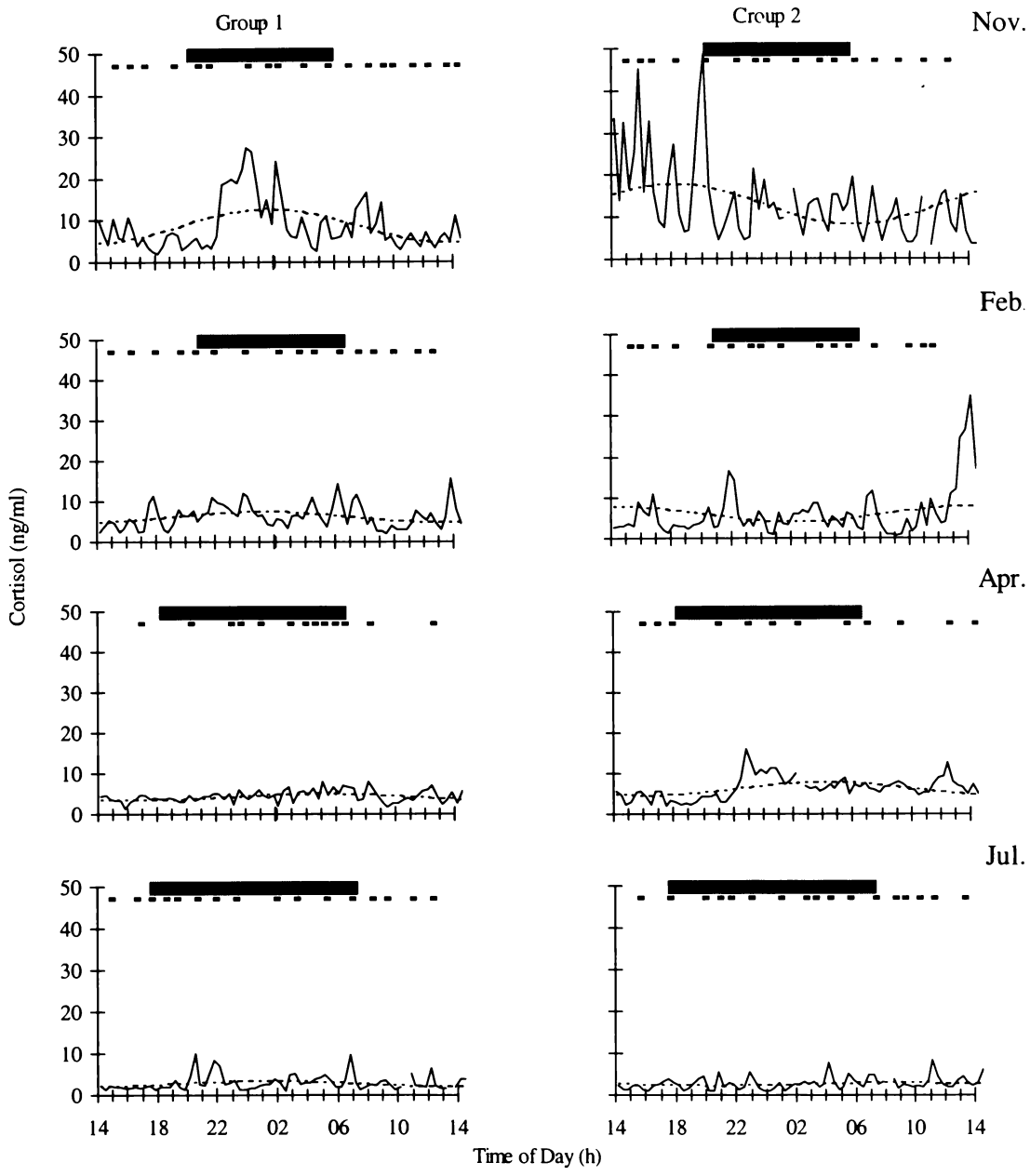


Figure 5.3 Seasonal effects on 24 h profiles of plasma cortisol collected from a representative stag from each group (1-2) at four different times of the year (Nov) November, (Feb) February, (Apr) April and (Jul) July. The occurrence of cortisol pulses as detected by cluster analysis are shown as points at the top of each graph. Significant circadian rhythms are represented by the best fitting sine curve (dashed line) for each data series derived from COSINOR analysis. The black bars at the top of each graph represent the period of darkness for each month.

	November	February	April	July
24 h Mean	12.5 ± 1.0^{a}	6.3 ± 1.0^{b}	4.0 ± 1.0^{b}	4.2 ± 1.0^{b}
Pulse:				
Frequency (pulses/h)	0.89 ± 0.03^{a}	0.82 ± 0.03^{ab}	0.72 ± 0.03^{b}	0.90 ± 0.03^{a}
Height (ng/ml)	17.1 ± 1.3^{a}	9.0 ± 1.3^{b}	5.7 ± 1.3^{b}	6.5 ± 1.3^{b}
Nadir (ng/ml)	7.5 ± 0.6^{a}	3.4 ± 0.6^{b}	2.8 ± 0.6^{b}	2.3 ± 0.6^{b}
Amplitude (ng/ml)	9.7 ± 0.8^{a}	5.3 ± 0.8^{b}	2.9 ± 0.8^{b}	4.1 ± 0.8^{b}
Width (min)	61.1 ± 2.6^{a}	62.6 ± 2.6^{ab}	69.5 ± 2.6^{b}	57.2 ± 2.6^{a}

Table 5.1 Seasonal effects on plasma cortisol concentrations (ng/ml) and cortisol pulse parameters (means ± S.E.M) derived from cluster analysis of 24 h cortisol profiles collected from six red deer stags at four different times of the year.Values with different superscripts are significantly different ($p \leq 0.05$) between months

Significant ($p < 0.05$) circadian rhythms were found in 9 of the 20 cortisol profiles suitable for analysis (Table 5.2). Another two profiles in February approached significance ($p = 0.051$ and $p = 0.061$) and were included in the overall analysis. Parameters of the fitted sine curves are also given in Table 5.2. The amplitude of the circadian rhythm when present tended to be higher in November than in February, April and July, although this difference was not significant ($p = 0.146$, overall mean 3.8 ± 1.4 ng/ml). The acrophase (highest point)of the circadian rhythm in cortisol secretion occurred at significantly different ($p < 0.05$) times of the day in April and February. While the times of the acrophase in November and July were not significantly different ($p > 0.05$) from either April or February.

Circadian rhythm	November	February	April	July
No. of animals*	4/6	2[†]/4	3/5	2/5
Amplitude (ng/ml)[+]	8.4 ± 2.5[a]	1.5 ± 2.5[a]	1.1 ± 2.5[a]	2.1 ± 2.5[a]
Acrophase (h:min)[⊕]	00:07 ± 2:29[ab]	17:44 ± 2:29[a]	06·42 ± 2:29[b]	01:36 ± 2:29[ab]

Table 5.2 Circadian effects on plasma cortisol concentrations derived from COSINOR analysis of 24 h cortisol profiles at four different times of the year (means ± S.E.M.).*No. of animals indicates stags exhibiting a significant ($p < 0.05$) circadian rhythm in plasma cortisol concentrations out of those stags with a complete 24 h profile (ie ≥ 72 samples). [+]Amplitude is the mean amplitude of the fitted sine curve expressed as ng/ml. [⊕]Acrophase is the mean time of day that the sine wave is maximal, expressed in hours and minutes. [†] In February the two animals had rhythms that approached significance ($p = 0.051$ and $p = 0.061$, respectively). Values with different superscripts are significantly different ($p < 0.05$) between months.

5.4.2 ACTH challenge

The mean cortisol concentrations following ACTH challenge for each of the four months are presented in Figure 5.4. The preinfusion cortisol concentrations were not significantly different ($p > 0.05$) between months (mean 4.6 ± 0.4 ng/ml). Peak cortisol concentrations following ACTH infusion were significantly higher ($p < 0.05$) in November (55.8 ± 2.7 ng/ml) and lower ($p < 0.001$) in April (33.7 ± 1.8 ng/ml) than those in February and July (48.7 ± 2.0 ng/ml and 45.4 ± 2.0 ng/ml, respectively).

The area under the cortisol response curve was significantly smaller in April (266.6 ± 15.3 ng/ml/190min) ($p < 0.05$) than at other times of year (February, 366.1 ± 15.3 ng/ml/190min; July, 340.7 ± 15.3 ng/ml/190min and November, 387.8 ± 21.2 ng/ml/190min).

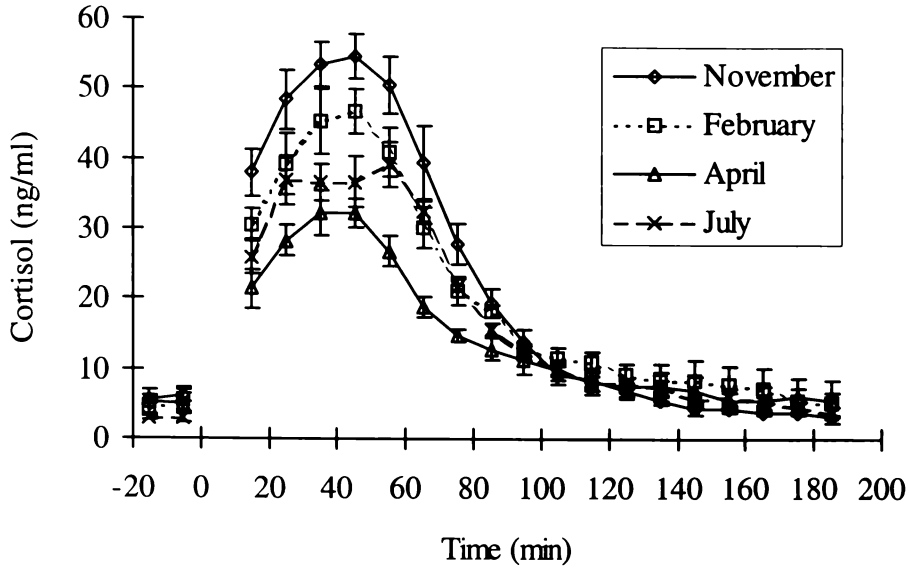


Figure 5.4 Seasonal effects on adrenocortical responses to $ACTH_{1-24}$ challenge (0.04 IU kg^{-1}) administered at time 0, to free-ranging red deer stags at pasture in November (n = 4), February (n = 5), April (n = 5) and July (n = 5). Values are shown as the monthly mean ± S.E.M.

5.4.3 Yarding stressor

The mean cortisol concentrations in response to the yarding stressor for each of the four months are presented in Figure 5.5. The concentration of cortisol in baseline samples taken before the yarding stressor did not differ significantly between months (mean 4.2 ± 0.5 ng/ml). There were no significant seasonal differences in the peak concentrations of cortisol following yarding (November 23.1± 5.6 ng/ml; February 20.7 ± 4.9 ng/ml; April 10.9 ± 5.6 ng/ml and July 9.7 ± 4.6 ng/ml), or in the area under the cortisol response curve (November 81.5 ± 32.2 ng/ml/70min; February 101.6 ± 27.9 ng/ml/70min; April 47.9 ± 32.2 ng/ml/70min and July 40.1 ± 26.6 ng/ml/70min).

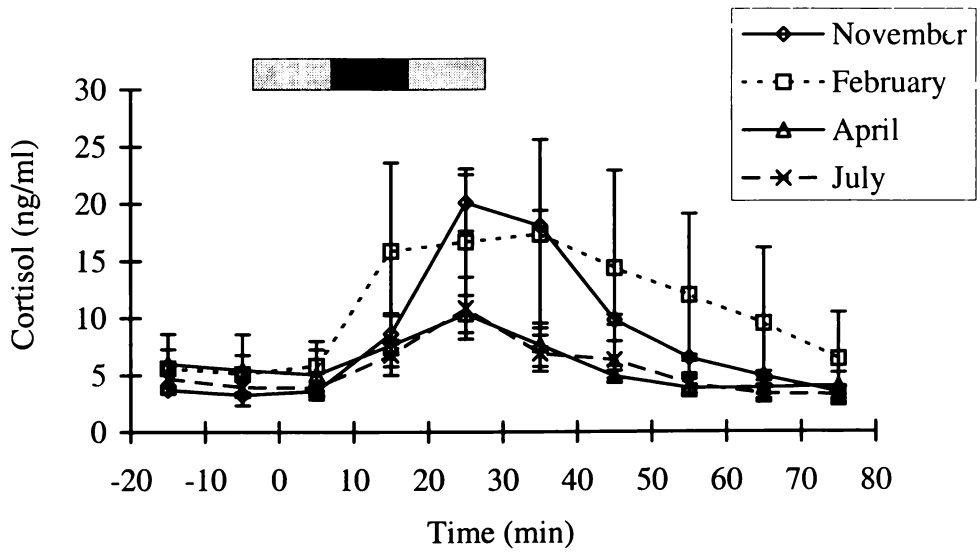


Figure 5.5 Seasonal effects on the adrenocortical response of free ranging red deer stags to a yarding stressor and subsequent recovery at pasture in November (n = 4), February (n = 5), April (n = 4) and July (n = 5). Grey bars indicate time spent moving to and from yards, the black bar indicates time spent in yards. Values are shown as the monthly mean ± S.E.M.

5.5 DISCUSSION

This study has shown that basal cortisol secretion in red deer stags is characterised by an episodic pattern of release from the adrenal gland, a feature common in other species (humans, Krieger *et al.* 1971; primates, Sarnyai *et al.* 1995; sheep, Fulkerson & Tang 1979). Significant pulses in cortisol concentration occurred on average every 72 min, a frequency (0.8 peaks/h) similar to that reported for Eld's deer (0.6 peaks/h, Monfort *et al.* 1993), and other ruminant species including cattle (0.5 peaks/h, Ladewig & Smidt 1989, Lefcourt *et al.* 1993), sheep (0.8-1.2 peaks/h, Fulkerson & Tang 1979) and non ruminant species such as the horse (0.6 peaks/h Irvine & Alexander 1994). This pulsatile release of cortisol during periods of normal undisturbed activity may function to prevent down regulation of the adrenal axis, thereby maintaining the ability of the axis to respond maximally to stressors.

Individual mean 24 h concentrations of plasma cortisol found in the present study (1.9 to 22.5 ng/ml) are similar to basal cortisol concentrations reported for hand-

reared Eld's deer (5.4-14.5 ng/ml; Monfort *et al.* 1993) and in previous studies using the DracPac remote sampling device on undisturbed red deer stags (5.4 - 22 ng/ml Ingram *et al.* 1994, 1997, Matthews *et al.* 1994, Carragher *et al.* 1997, Waas *et al.* 1997) as well as in stags blood sampled immediately after being shot dead while undisturbed at pasture (5.7 ± 3.7 ng/ml, Smith & Dobson 1990). These basal concentrations are generally lower than those reported for red deer in studies where animals have been physically or chemically restrained prior to blood sampling (6 - 27 ng/ml, Jopson *et al.* 1990); (18 - 27 ng/ml, Goddard *et al.* 1994); (17 - 55 ng/ml, Bubenik & Bartos 1993). This indicates that in previous studies, restraint artefacts have been a potential confounding factor in the assessment of adrenal activity and function in red deer.

The presence of a circadian rhythm in plasma glucocorticoid concentrations is well established in humans (Weitzman *et al.* 1971) and rodents (Keller-Wood & Dallman 1984) and involves an anticipatory rise in cortisol prior to the activity phase, which is typically entrained by circadian rhythms in feeding (Saito *et al.* 1989) and resting (Born *et al.* 1997). In ruminants, contradictory reports exist about the presence of circadian rhythms in basal plasma cortisol concentrations. For example, in cattle several authors have reported a circadian rhythm in cortisol concentrations (Hays *et al.* 1975, Fulkerson *et al.* 1980, Thun *et al.* 1981, Lefcourt *et al.* 1993) whereas Hudson *et al.* (1975), failed to detect any circadian rhythm. Such rhythms in cortisol concentrations have not been identified in white-tailed deer (Bubenik *et al.* 1983), rusa deer (van Mourik & Stelmasiak 1984) or Eld's deer (Monfort *et al.* 1993), though the existence of a circadian rhythm in the later study could not be ruled out as the animals were blood sampled for only 10 h per day. The deer in these studies were all subjected to either handling or indoor housing, factors known to attenuate circadian rhythms in horses (Irvine & Alexander 1994). In the present study, a significant circadian rhythm in plasma cortisol concentrations was found in half of the 24 h profiles suitable for analysis. Amplitudes were generally small, the exception being November, when a sustained period of agonistic behaviour in one group (unpublished data) may have contributed to increased amplitudes.

Where reported, circadian rhythms are typically of low amplitude in ruminants (e.g. cattle, 1-1.4 ng/ml, Thun *et al.* 1981, Lefcourt *et al.* 1993). At pasture, ruminants such as sheep, cattle and deer graze and rest in bouts throughout the day and night (Gates & Hudson 1983, Kilgour & Dalton 1984). Thus, they may not have the same cues available for entrainment of a circadian rhythm compared with non-ruminants or ruminants maintained indoors on fixed feeding schedules. While there is some evidence from our study for circadian variability in cortisol concentrations in red deer at pasture, this rhythm is of low amplitude and potentially easily disrupted by stress associated with handling and blood sampling or social and environmental factors (Irvine & Alexander 1994). Further studies would be required to determine conclusively if circadian rhythms were a fundamental feature of HPA axis activity in red deer stags.

Seasonal rhythms in activity, behaviour, metabolism and secretion of hormones are well documented in cervid species (Lincoln *et al.* 1970, Loudon & Brinklow 1992, Suttie *et al.* 1992, Asher *et al.* 1996). In the present study significant seasonal rhythms were observed in adrenal cortisol 24 h secretory dynamics in red deer stags. The decline in pulse frequency during the rut is in contrast to the lack of a seasonal pattern found in Eld's deer (Monfort *et al.*, 1993) and may reflect fundamental differences between the two species of deer (temperate vs. tropical habitat, autumn vs. spring breeding). The role androgens might play in modulating the pulsatile nature of HPA axis activity in red deer has yet to be investigated; however, androgens have been shown to suppress corticotrophin releasing hormone levles within the paraventricular nucleus (Bingaman *et al.* 1994) suggesting a central site of action (Handa *et al.* 1994a).

November (southern hemisphere) is a unique time of year for red deer stags. It represents a degree of sexual quiescence uncommon in many other domestic ruminants. Secretion of lutinising hormone (LH) from the pituitary and testosterone from the testes is negligible (i.e. pulse frequency of LH and testosterone is less than one pulse per 24h and basal concentrations of LH and testosterone are below detectable concentrations (Suttie *et al.* 1992)). With no significant testicular activity, stags behave similarly to castrated animals (Lincoln *et al.* 1970). Major changes also occur in appetite, feed utilisation and fat

accretion (Asher *et al.* 1994). The increase in cortisol pulse height, pulse nadir and pulse amplitude in November indicates that there is a shift in the regulation of the HPA axis during Spring. Changes in height and amplitude reflect to some extent the increased responsiveness of the adrenal gland to ACTH seen at this time of year (Suttie *et al.* 1995, Cassidy 1996). However, the threefold increase in cortisol pulse parameter values compared with that seen during the rut would suggest that the adrenal gland changes are accompanied by changes in endogenous ACTH secretion. Due to its rapid degradation in blood, ACTH could not be measured in remotely collected samples. However, male Soay sheep which display similar seasonal rhythms in basal cortisol concentrations and adrenal responsiveness to ACTH also show a marked seasonal variation in endogenous ACTH secretion with the peak concentrations occurring during the seasonal peak in cortisol concentrations (Ssewannyana *et al.* 1990). The increase in nadir also implies an up regulation of the central drive to the HPA axis in conjunction with a down regulation of the glucocorticoid negative feedback system controlling the axis.

The increase in cortisol pulse parameters in November translated into greater mean 24 h cortisol concentrations which were significantly higher than those at other times of year. Seasonal changes in cortisol secretion have been reported in males of some other seasonally breeding deer species. Cortisol concentrations have been reported to decline during the rut in whitetail deer (Bubenik *et al.* 1983), reindeer (Nilssen *et al.* 1985) and axis deer (Chapple *et al.* 1991), while other studies have reported increased concentrations during the rut for red deer (Feher *et al.* 1994), or no change in reindeer (Ringberg 1979) and Eld's deer (Monfort *et al.* 1993). The metabolic and reproductive implications of a seasonal rhythm in basal cortisol concentrations in red deer stags are discussed below.

Alternatively, the decline in basal cortisol concentrations over the course of the experiment may result from a process of habituation to the remote blood sampling procedure. However, subsequent experiments (Chapter 6,7) conducted in November and April using red deer stags naive to the blood sampling procedure on both occasions, have revealed similar seasonal differences in cortisol concentrations to those found in the present study.

Cortisol concentrations and the temporal pattern of release from the adrenal gland in response to ACTH challenge were similar to those previously reported for red deer stags using the DracPac technique (Ingram *et al.* 1997). The maximum cortisol concentrations were within the range reported for red deer following ACTH challenge (males: 26 to 100 ng/ml, Bubenik & Bartos 1993, Ingram *et al.* 1997, females: 40 to 90 ng/ml, Jopson *et al.* 1990, Goddard *et al.* 1994) and following routine handling procedures in our facility (males: 25 to 70 ng/ml, Matthews & Cook 1991, Ingram *et al.* 1994, 1997, Matthews *et al.* 1994, Carragher *et al.* 1997). In the present study, red deer stags displayed significant seasonal variation in response to ACTH challenge. Maximal responses were higher in November (spring) and declined to a minimum in April (mid rut, autumn). Recent studies of adrenal responsiveness in red deer stags (Suttie *et al.* 1995, Cassidy 1996) have shown a similar seasonal rhythm with greater plasma cortisol responses to ACTH challenge during spring/summer compared with the breeding season and winter months.

Significant elevations in plasma cortisol concentrations were seen in response to a standardised acute stress (yarding) at all times of the year. The amplitude of the cortisol response to yarding in November and February was twice that seen in April and July, though this seasonal variation was not statistically significant due to the large variability seen in individual responses. Peak cortisol concentrations in November and February, however, were similar to concentrations reported in an earlier study on stags, carried out in December (23 ng/ml, Carragher *et al.* 1997), using the DracPac blood sampling technique and a similar yarding challenge. The generally lower cortisol responses in April and July would suggest either a reduced capacity of the HPA axis to respond to stress as supported by the ACTH challenge data, or a reduction in the perceived stressfulness of the yarding procedure possibly mediated via either habituation to the challenge or the attenuating effects of testosterone on fearfulness (Boissy & Bouissou 1994).

Compared with other routine handling procedures that have been evaluated using the DracPac technique, such as restraint (Ingram *et al.* 1994, Carragher *et al.* 1997) removal of growing antler (Matthews *et al.* 1994) and transport (Waas *et al.*

1997), the increase in cortisol secretion following yarding was of lessor amplitude and of short duration indicating that the procedure was potentially less stressful.

It is evident from this study that red deer stags have a strong seasonal rhythm in HPA axis activity and responsiveness. The mechanism by which this change occurs has yet to be determined in red deer, though these changes closely follow seasonal rhythms in testicular function (Suttie *et al.* 1992) and growth rates (Kay 1979). Testosterone concentrations which peak in stags during the rut (Suttie *et al.* 1992), can inhibit cortisol secretion directly by influencing steroidogenic pathways involved in the synthesis of cortisol in the adrenal cortex (Hornsby 1982, Miller 1988). It is possible that the hypertrophy and hyperpalsia of the adrenal cortex seen in rutting wild red deer stags (Kapp 1989) and white tailed deer stags (Hoffman & Robinson 1966) is in response to the reduced ability of the adrenal to produce cortisol at this time.

In addition, androgens can influence HPA axis activity by competing with cortisol for binding sites on carrier proteins such as corticosteroid binding globulin (CBG) (Bradley & Stoddart 1992) and plasma albumin (Ward *et al.* 1992). Androgens can also suppress hepatic synthesis of CBG (Gala & Westphal, 1965) effectively increasing unbound concentrations of cortisol. This in turn would increase negative feedback to the HPA axis, reducing HPA axis activity and trophic drive to the adrenal cortex. A further mechanism by which androgens can influence HPA axis activity is by binding to androgen receptors in regions of the central nervous system such as the hippocampus known to modulate the HPA axis (Handa *et al.* 1994a).

The seasonal rhythm in plasma cortisol concentrations found in the present study also correlates with the seasonal cycle in growth in the red deer stag, with higher concentrations of cortisol during the period of weight gain in November and reduced concentrations during periods of weight loss during the rut. Interestingly, humans suffering from Cushing's syndrome (chronic hypercortisolemia) show clinical signs of obesity whereas adrenocortical deficiency (chronic hypocortisolemia) in humans is characterised by weight loss (Hauner *et al.* 1987).

The dramatic increase in weight during the spring and early summer and the higher levels of plasma cortisol coincide with elevated insulin concentrations and the development of insulin resistance in red deer stags (McMahon *et al.*1997). It is well established that glucocorticoids stimulate feeding behaviour and insulin secretion (Dallman *et al.* 1995), and that cortisol excess can cause insulin resistance and obesity in humans (Brandes 1977). Cortisol promotes differentiation of adipocyte precursors into adipocytes and stimulates lipogenesis in the presence of insulin (Grégoire *et al.* 1991), and may have a role to play in the increased appetite, feed utilisation and fat accretion at this time of year in stags.

The period of the rut, with its increased aggression (Suttie1985) and pronounced weight loss (Kay 1979), could be considered a period of increased social and nutritional stress. Chronic social stress has been reported to reduce plasma cortisol concentrations in female red deer (Goddard et al. 1994), yearlings (Hanlon et al. 1995) and stags (Chapter 7). While prolonged periods of weight loss in humans (over 26 weeks) resulted in a significant decrease in concentrations of cortisol and cortisol-binding globulin but no change in the concentration of free cortisol (Yanovski *et al.* 1997). In addition, nutritional stress is often associated with a metabolic disorder termed fatty liver syndrome. Red deer stags during the rut in the wild, have a high incidence (80%) of fatty liver syndrome (Kapp *et al.* 1989a) which may impair liver function reducing the synthesis of CBG (Veldman & Meinders 1996) and can directly inhibit steroidogenesis by limiting the availability of cholesteryl esters used in the synthesis of steroid hormones (Morrow et al. 1979, Nakagawa et al. 1997).

Glucocorticoid excess stimulates bone resorption and inhibits bone formation resulting in accelerated bone loss (Kleerekoper *et al.* 1997). Deer undergo skeletal bone loss during antler growth in spring (Hillman *et al.* 1973). The elevated concentrations of cortisol during the period of new antler growth in the present study and during antler mineralisation in late summer (Suttie *et al.* 1995) suggests a role for this steroid in red deer new antler growth.

The inhibitory influence of the HPA axis upon the reproductive axis (reviewed in Rivest & Rivier 1995) and immune function (reviewed in Munck *et al.* 1984) is

well established in many animals. Therefore, it may be advantageous for stags during the breeding season, when inter-male competition for females is intense, to have reduced activity and responsiveness of the HPA axis as a strategy to maintain reproductive and immune competence in the face of increased physical and psychological stress.

In summary, the current data demonstrate that there is episodic activity of the HPA axis. There are also strong seasonal effects on HPA axis activity and responsiveness to ACTH challenge in red deer stags. November, a period of reproductive quiescence, new antler growth and rapid weight gain, is associated with higher mean plasma cortisol concentrations and a greater responsiveness to ACTH. In contrast, the breeding season is associated with lower adrenal activity and responsiveness. The mechanisms involved have yet to be elucidated for red deer, however the inhibitory actions of androgens, variations in metabolic demand and exogenous factors such as chronic social/nutritional stress may be important factors. This study also highlights the advantages of remote blood sampling technology in obtaining undisturbed measurements of HPA axis activity and functioning without the confounding effects of repeated handling and restraint stress. Information which is essential before reliable physiological measures of stress can be determined.

CHAPTER 6 THE ACTIVITY AND FUNCTION OF THE HPA AXIS IN CASTRATED AND ENTIRE RED DEER STAGS (*CERVUS ELAPHUS*) DURING THE BREEDING SEASON.

6.1 ABSTRACT

Red deer (*Cervus elaphus*) stags exhibit a well documented annual cycle in reproductive function, growth and antler development. Recent studies (Suttie *et al.* 1995, Cassidy 1996, Ingram *et al.* 1997, 1999) have also demonstrated a marked seasonal rhythm in the activity and functioning of the hypothalamic-pituitary adrenal (HPA). In stags, the seasonal nadir in basal glucocorticoid concentrations and in adrenal responsiveness to ACTH is associated with the seasonal peak in reproductive activity. The aim of the current study was to determine the role of gonadal steroids in modulating the seasonal rhythm of HPA axis activity and function in the red deer stag. This was achieved by comparing the basal secretion of cortisol in castrated (n=6) and entire (n=6) male deer during the breeding season. Differences in functioning of the HPA axis were quantified by measuring changes in plasma cortisol in response to administration of ACTH $_{1\text{-}24}$ (0.04 IU kg^{-1} live weight), CRH (25 ng kg^{-1} live weight) and dexamethasone (37 μg kg^{-1} live weight). The plasma steroid binding capacity (i.e. free cortisol concentrations) and the clearance rate of cortisol from circulation were also measured.

Mean 24 h basal plasma cortisol concentrations tended to be higher ($p < 0.06$) in castrates (6.22 ± 0.84 ng/ml) than in entire stags (3.95 ± 0.75 ng/ml). Several cortisol pulse parameters (pulse height, amplitude and area under the curve) were greater ($p < 0.05$) in castrated stags. Circadian rhythms in plasma cortisol concentrations were seen in 3 entire stags and 5 castrates though no difference in amplitude (overall mean: 2.33 ± 0.54 ng/ml) or acrophase (overall mean: 07:11 ± 0:37 h:min.) was seen between castrated and entires. The messor was higher ($p < 0.05$) in castrates (6.09 ± 0.85 ng/ml) than entire stags (3.80 ± 0.60 ng/ml).

Adrenal responsiveness to ACTH, as measured by the peak cortisol response following ACTH challenge, was higher ($p < 0.01$) in castrated (53.1 ± 4.13 ng/ml) than entire (31.24 ± 4.25 ng/ml) animals. The area under the cortisol response curve was also greater in castrates (338.47 ± 28.3 ng/ml/100min) than entire stags (183.25 ± 41.8 ng/ml/100min). The ratio of free to total cortisol was lower ($p < 0.001$) in castrated (8.93 ± 0.39 %) than entire stags (12.91 ± 0.94 %) during periods of undisturbed basal activity and during peak cortisol responses to ACTH challenge (17.89 ±1.03 and 24.01 ± 2.45 %, respectively). Castrated and entire stags did not differ significantly in their cortisol responses to either CRH or dexamethasone challenge.

The higher level of HPA axis basal activity and adrenal responsiveness to ACTH seen in castrated compared with entire stags during the breeding season suggests that androgen suppression of HPA axis function constitutes a major part of the regulatory changes responsible for the seasonal rhythm in HPA axis function in stags. Modulation of HPA axis function by testicular steroids appears to occur primarily at the level of the adrenal and not the pituitary as differences were found in response to ACTH but not CRH or dexamethasone challenge. A reduction in HPA axis activity and responsiveness during the breeding season when inter male competition for females is intense may serve to reduce disruption of reproductive and immune competence at a time of increased physical and psychological stress.

6.2 INTRODUCTION

Changes in the activity and functioning of the hypothalamic-pituitary-adrenal (HPA axis) are routinely used to quantify stress in farm animals (Barnett & Hemsworth 1990, Fraser and Broom 1990). However, in male red deer, basal cortisol concentrations and adrenal responsiveness to ACTH change with season (Suttie *et al.* 1995, Cassidy 1996, Ingram *et al.* 1997, 1999, Chapter 5). This seasonal rhythm in HPA axis activity and function appears to coincide with the well documented annual cycle in reproductive function, growth and antler development (Clutton-Brock *et al.* 1982, Suttie *et al.* 1995). In the male red deer, spring and early summer represents a period of reproductive quiescence (stags are infertile and behave in a manner similar to castrates), new antler growth and rapid liveweight gains. Spring and early summer also represent a time of increased basal plasma cortisol concentrations (Chapter 5) and a greater responsiveness to ACTH in the red deer stag (Suttie *et al.* 1995, Cassidy 1996, Chapter 5). During the autumn breeding period (rut), stags have high levels of circulating testosterone (Suttie *et al.* 1992), increased aggression (Suttie1985), and a reduced voluntary food intake (Loudon *et al.* 1989) which results in pronounced weight loss (Kay 1979). The rut and winter period is also associated with lower basal plasma cortisol concentrations (Ingram *et al.* 1999, Chapter 5) and a reduced adrenal responsiveness to ACTH compared with spring and early summer (Suttie *et al.* 1995, Cassidy 1996, Ingram *et al.* 1999, Chapter 5).

The hypothalamic-pituitary-gonadal (HPG) axis has been shown to play a critical role in modulating the differences between sexes, with estrogens enhancing and androgens diminishing HPA axis basal activity and responsiveness to stress (e.g. rat: Critchlow *et al.* 1963, Handa *et al.* 1994b). Differences in the responses of the HPA axis between males and females or entire and gonadectomised animals has been reported in a number of species including human (Seeman *et al.* 1995), rats (Rivier 1993, Handa *et al.* 1994b), mice (Gaillard & Spinedi 1998)(reviewed in Handa *et al.* 1994a, Vamvakopoulos & Chrousos 1994). Compared to males, females generally have higher basal levels of plasma glucocorticoids, greater adrenal responsiveness to ACTH and a higher and more prolonged elevation of glucocorticoids in response to

stress, while ovariectomised females and castrated males have a response intermediate to entire males and females (e.g. rat: Handa *et al.* 1994a).

In both males and females, gonadal steroids have been shown to modulate the activity of the HPA axis at virtually all levels of the axis (Canny *et al.* 1999). There are sex-related differences in the levels of GR and MR glucocorticoid receptors in the hippocampus, hypothalamus and pituitary, which are dependent on gonadal steroids (Turner & Weaver 1985, Turner 1990, MacLusky *et al.* 1996). In female rats, hyperactivation of the HPA axis after stress compared with males is due in part to the effects of estrogens feedback (Burgess & Handa 1992). This estrogen induced decline in receptor function has been reported to occur within the hippocampus (Burgess & Handa 1992) and pituitary (Turner 1990) of the female rat. In male rats, high physiological levels of testosterone have been reported to increase GR receptor binding in the medial preoptic area, with no effect on MR receptor binding or on GR receptor binding in other regions (Viau & Meaney 1996). Corticosterone implants into the medial preoptic area of rats inhibit the HPA axis responses to restraint stress (Viau & Meaney 1996) and thus play a significant role in the negative feedback regulation of the HPA axis.

The expression of CRH and AVP in the hypothalamus also exhibits sex-related differences that are due in part to gonadal steroids. In the human female, have a direct stimulatory effect on CRH gene expression (e.g. Vamvakopoulos & Chrousos 1993). In female rats estrogens enhances CRH levels within the paraventricular nucleus (PVN) (Haas & George 1988, Paulmyer-Lacroix *et al.* 1996). Conversely, androgens have been reported to decrease arginine vasopressin (AVP) levels in the median eminence of the male rat (Viau & Meaney 1996). Androgens are also believed to indirectly inhibit CRH levels within the PVN by binding to regions of the brain rich in androgen receptors which project to the PVN and influence HPA axis activity (e.g. hippocampus, medial preoptic nucleus, bed nucleus of the stria terminalis, and lateral septum) (Handa *et al.* 1994a).

In addition to these centrally mediated effects, gender differences in the HPA axis are also seen at the level of the anterior pituitary and in the size, structure and steroidogenic potential of the adrenal glands (reviewed by Parkes & Deanesly

1966). Estrogens have been reported to increase cortisol production from the adrenal (Kitay *et al.* 1963), and anterior pituitary responsiveness to CRH (Coyne & Kitay 1971, Gallucci *et al.* 1993). The adrenal responsiveness to ACTH, in terms of glucocorticoid production, is greater in female and castrated male rats or cattle than in intact males (Kitay 1961, 1963, Bass *et al.* 1982, Verkerk & Macmillan 1997). Androgens have been shown to inhibit the activity of specific enzymes in the adrenal cortex involved in the synthesis of glucocorticoids in a number of species including humans (Miller 1988, Vermesh *et al.* 1988), rats (Carsia *et al.* 1983), hamsters (Gaskin & Kitay 1971) and cattle (Hornsby 1982, Carsia *et al.* 1983).

In male rats, the removal of gonadal steroids by castration increases levels of CRH and numbers of CRH containing cells within the PVN and ACTH and glucocorticoid response to physical and psychological stressors (Handa *et al.* 1994b). At least 10 days is required following castration before increases are apparent in the rat (Bingaman *et al.* 1994, Handa *et al.* 1994a). Increases in CRH levels within the PVN following castration are not accompanied by changes in anterior pituitary responsiveness to CRH in rats (Handa *et al.* 1994a) implying that regulatory changes occur at levels other than the pituitary.

Other mechanisms may also be involved in the androgen regulation of HPA axis activity. Since plasma concentrations of glucocorticoids represent a balance between adrenal secretion and the rate of clearance from circulation, lower glucocorticoid concentrations in entire males may represent an increase in the metabolic clearance rate of cortisol. Gender differences in the metabolic clearance rate of glucocorticoids have been reported in the guinea pig (*Cavia porcellus*) as measured by clearance rate following steady state infusions of radioactive cortisol (El Hani *et al.* 1980, Greiner *et al.* 1976). The metabolic clearance rate is higher in males than in females or castrates (El Hani *et al.* 1980). Similarly, treating female guinea pigs with testosterone results in an increase in the metabolic clearance rate of cortisol (El Hani *et al.* 1980). The rate of removal of cortisol from the circulation can be estimated by monitoring the decline in cortisol concentrations following the peak response to ACTH or following dexamethasone challenge. The decline in cortisol in these situations primarily reflects the metabolism and removal of cortisol from

the circulation as further endogenous release of cortisol is normally suppressed (Verkerk & Macmillan 1997).

Gonadal steroids influences on cortisol binding capacity of plasma may also contribute to gender differences in cortisol concentrations. This binding capacity is largely determined by the concentrations of corticosteroid-binding globulin (CBG), which binds cortisol with much higher affinity and specificity than albumin and is the major regulator of cortisol bioavailability (Hammond 1997). Estrogens enhance plasma corticosteroid-binding capacity by increasing the hepatic synthesis of corticosteroid-binding globulin (CBG) (Coe *et al.* 1986). Increased binding of glucocorticoids to CBG will decrease the availability of circulating glucocorticoids for metabolic clearance (Bright 1995), receptor mediated inhibitory feedback and bioavailability to target tissues (Coe *et al.* 1986). An inverse relationship appears to exist between androgen concentrations and CBG binding capacity. Mataradze *et al* (1992) reported a 40-50% increase in CBG concentrations following castration of male rats with CBG concentrations returning to normal after testosterone treatment. Also, the males of seasonally breeding species such as the marsupial sugar glider (*Petaurus breviceps*) and the red-tailed phascogale (*Phascogale calura*) exhibit an androgen dependent fall in CBG concentration during the breeding season (McDonald *et al.* 1981, Bradley 1987, Bradley & Stoddard 1992). Androgens may reduce the corticosteroid-binding capacity of plasma by suppressing the hepatic synthesis of CBG (Gala & Westphal 1965) or by competing with cortisol for binding sites on corticosteroid carrier proteins such as CBG and albumin (Ballard 1979, Hammond 1990).

The measurement of HPA axis activity and function is readily confounded by the stress associated with handling and restraint of animals with traditional blood sampling techniques (Seal *et al.* 1972, Hattingh *et al.* 1988). This effect is further exacerbated in wild or semi-domesticated animals or in species with flighty natures such as red deer (Ingram *et al.* 1994, 1997, Carragher *et al.* 1997). In order to eliminate factors such as handling stress, which may be further confounded by the differences in fearfulness exhibited by castrated males and intact male conspecifics (Vandenheede & Bouissou 1996), a remote blood sampling device (Ingram *et al.* 1999) was used to obtain blood samples from undisturbed animals at pasture.

The aim of the current study was to determine the role of gonadal steroids in modulating the seasonal rhythm of HPA axis activity and function in the red deer stag as seen in Chapter 5. This was undertaken by comparing basal activity and the functioning of components of the HPA axis in castrated and entire red deer stags during the breeding season. We sought to quantify differences in adrenal responsiveness to ACTH, a parameter that exhibits a strong seasonal rhythm opposite to that of the seasonal rhythm in reproductive function (Ingram *et al.* 1999, Chapter 5). The role of the pituitary in gonadal steroid regulation of the HPA axis was assessed by quantifying the responsiveness of the anterior pituitary to a CRH challenge, a test that to our knowledge has not been performed previously on any deer species. In addition, glucocorticoid receptor mediated negative feedback control of the HPA axis was quantified in both castrated and entire stags by comparing the suppression in cortisol secretion following administration of dexamethasone (a glucocorticoid agonist). The role of other factors in the regulation of the HPA axis and cortisol concentrations such as changes in plasma steroid binding capacity and the clearance rate of cortisol from circulation following ACTH and dexamethasone challenge were also measured and compared between castrates and entires.

6.3 MATERIALS AND METHODS

The following experimental protocol was approved by both the University of Waikato and Ruakura Animal Ethics Committees.

6.3.1 Animals

Thirty six 18-30 month-old red deer stags were maintained in six groups (n = 6 animals/group) at the Ruakura Agricultural Centre, Hamilton, New Zealand (37°46'S, 175°20'E). Animals in three of the groups (n = 18) were surgically castrated at least 48 days prior to the start of the experiment. The other three groups (n =18) were kept as entires. Each group was kept in separate 0.25 hectare paddocks during the experiment with *ad libitum* access to pasture (ryegrass-white clover) and water. A degree of spatial separation between groups was achieved

by using alternate paddocks on both sides of a central race and alternating neighbouring groups between castrated and entires. Two animals from each group served as the experimental animals.

6.3.2 Procedure and remote blood sampling

The Experiment was conducted over two consecutive 1 week periods (weeks 1 and 2) during the southern hemisphere breeding season (rut). One stag in each group of castrates and entires was was blood sampled each week (6 per week). At the start of each week, all animals were weighed and the Experimental animals to be remotely blood sampled that week, were catheterised and fitted with a canvas and leather backpack as described in Chapter 5. Basal activity of the HPA axis was determined by sampling for 24 h on the second day of each week. On this day all groups were penned between 0600 and 1030 h and each of the 6 catheterised animals fitted with a battery powered remote blood sampling device (DracPac, ABWRC, AgResearch, Ruakura Agricultural Centre, Hamilton, N.Z and Engineering Development Group, HortResearch, Ruakura Agricultural Centre, Hamilton, N.Z.). They were then returned to their respective paddocks and allowed to recover from the effects of handling for a minimum of 2.5 h before remote blood sampling commenced at 1300 h. The DracPac was programmed to collect samples over 24 h (72 x 20 min) continuously at a rate of 15 ml per hour. The procedure and equipment are as described in Chapter 5. At the end of this 24 h collection period (1300 h, Day 3 of each week), the animals were returned to the yards and the DracPac and blood samples were removed from the backpack.

The glucocorticoid responses to remotely administered ACTH and CRH challenges were determined on the 4th day of each week. All groups were penned between 0600 and 1030 h and each of the 6 catheterised animals were again fitted with a DracPac. Animals were then returned to their respective paddocks for a period of recovery (minimum of 2.5 h) before remote blood sampling commenced at 1300 h. Two 10 min baseline samples (30 ml/h) were then automatically collected before 2 ml of physiological saline containing ACTH $_{1\text{-}24}$ (0.04 IU kg^{-1} live weight; Synacthen; Ciba NZ, Auckland, NZ) was infused into the jugular vein over 5 min starting at 1320 h (time 0, Figure 6.2). Blood was then drawn from the animal through the

blood lines to a waste tube for 5 min to clear the blood lines of saline. Following the ACTH infusion and flushing of blood lines (total time 10 min) twelve 10 min blood samples (30 ml/h) were then collected over 120 min. Two hours after the ACTH challenge, at 1530 h, 2 ml of physiological saline containing, Bovine CRH (25 ng kg^{-1} live weight, Sigma), Bovine Serum Albumin (10 mg ml^{-1}, Sigma) and L-Ascorbic Acid (1 mg ml^{-1}, Sigma) was infused over 5 min into the jugular vein of the animals followed by flushing of the lines as per the ACTH challenge. Ten 10 min blood samples (30 ml/h) were then collected before returning the animals to the yards where the blood samples, sampling equipment and harnesses were removed from the deer (not catheters).

Changes in glucocorticoid negative feedback of the HPA axis were measured on Day 6 of each week by administering a dexamethasone suppression test. All groups were brought into the yards (0930-1030h), and held in indoor pens. Blood samples were collected manually starting at 1230 h with three baseline blood samples (10 ml) collected at 15 min intervals. Immediately following the third sample (1300 h), Dexamethasone sodium phosphate (0.037 mg kg^{-1} live weight, Dexone-5, Bomac Laboratories Ltd, Auckland, New Zealand) was administered via the catheter which was then flushed with saline. A further 7 blood samples (10 ml) at 10 min intervals followed by two at 15 min intervals were collected. After the final sample was collected (1600 h) catheters were removed from the 6 animals and a prophylactic dose of antibiotic (Tripen LA, Ethical Agents Ltd., Auckland, N.Z) administered (*i.m.*).

6.3.3 Assays

6.3.3.1 Total plasma cortisol analyses

All blood samples were centrifuged (1200 g for 15 min) after removal from the backpacks (remotely collected samples) or immediately after manual collection, and the plasma stored at -20°C until assayed for cortisol. Plasma cortisol concentrations were determined in duplicate, after extraction in ethyl acetate, by an ^{125}I radioimmunoassay method with polyethylene glycol separation (Ingram *et al.*, 1994). A standard curve, using charcoal stripped deer plasma, was also extracted

and used to calculate the concentration of cortisol in individual plasma samples. The cross-reactivity of the antiserum with 11 deoxycortisol was 5.7% and cortisone 1.2%. The inter and intra-assay co-efficient of variations for spiked deer plasma controls of known low (5 ng/ml), medium (20 ng/ml) and high (50 ng/ml) cortisol concentrations were 7.2 % and 13.7 % (low), 9.8 % and 10.3 % (medium), 11.3 % and 11.9 % (high), respectively. Assay sensitivity was 0.85 ng/ml.

6.3.3.2 Free cortisol analyses

The unbound (free) cortisol fraction in plasma was determined in both castrated and entire stags using samples with low (basal) and high (peak) cortisol concentrations. Low samples (Low) were from the baseline period prior to ACTH challenge and high samples (High) corresponded to the period of peak cortisol responses following ACTH challenge. The unbound cortisol fraction in plasma was quantified in undiluted red deer plasma by the centrifugal ultrafiltration dialysis method of Hammond *et al.* (1980). In brief, the percentage of free cortisol was determined by incubating 450 µl of plasma at 37 °C in 95% O_2 : 5 % CO_2 incubator for 30 min with ^{3}H-cortisol (3 x 10^5 dpm) and ^{14}C-glucose (12 x 103 dpm). Duplicate 200 µl aliquots were then pipetted into a dialysis tube comprising of a small glass tube with dialysis membrane covering one end. The dialysis tube was then placed inside a scintillation vial containing discs of cellulose filter paper. The scintillation vial was then centrifuged at 3000g for 1 h at 37 °C. Following centrifugation the dialysis tube was removed from the scintillation vial and 30 µl of serum remaining in the dialysis tube (unfiltered) was placed in a second scintillation vial. The quantity of ^{3}H-cortisol and ^{14}C-glucose present in the filtered plasma (which had passed through the dialysis membrane) and in the 30 µl aliquot of unfiltered plasma was then determined by adding scintillation liquid (3 ml, ACS, Amersham) and counting in a spectrophotometer (β counter) adjusted for the simultaneous measurement of ^{3}H and ^{14}C. The ^{14}C-glucose is unbound in plasma and is used as an internal standard to correct for differences in the volume of the filtered sample. As ^{3}H-cortisol binds to CBG and albumin only the proportion of unbound ^{3}H-cortisol is in equilibrium on either side of the membrane. Therefore, the following equation can then be used to calculate the proportion of free cortisol in the sample:

$$\text{Proportion of Free Cortisol} = \frac{^{3}\text{H - cortisol (dpm)}}{^{14}\text{C - glucose (dpm)}} \div \frac{^{3}\text{H - cortisol (dpm)}}{^{14}\text{C - glucose (dpm)}}$$

(filtered) *(unfiltered)*

The absolute concentration of free cortisol (ng/ml) was derived by multiplying the proportion of free cortisol in the sample by the total cortisol concentration obtained from the plasma cortisol assay. Inter and intra-assay co-efficient of variations for the analysis of free cortisol obtained from an assay control of pooled red deer plasma samples were 3.3 and 13.7 %, respectively.

6.3.4 Statistical analysis

6.3.4.1 Pulse detection

Discrete pulses in cortisol secretion were quantified using a statistically based peak detection algorithm termed cluster analysis (Veldhuis & Johnson 1986) (see Chapter 5 for details). A cluster size of one point for the nadir and one point for a peak was used along with a constant 15 % coefficient of variation, a minimum peak size of 0.85 ng/ml and a pooled t statistic of 1 to limit the false peak detection rate to approximately 10 % (Veldhuis & Johnson, 1986). The pulse parameters measured for each 24 h profile were pulse frequency, pulse height, pulse amplitude, nadir and peak width.

6.3.4.2 Circadian rhythm detection

Circadian rhythms in plasma cortisol concentrations were detected using a single cosinor model developed for Microsoft Excel (Microsoft Corp., USA) by Bourdon *et al.* (1995) (see Chapter 5 for details). The mean of which the circadian rhythm fluctuates around (mesor), the amplitude of the circadain rhythm and the time of day at which the circadian rhythm was at its peak (acrophase) were obtained using this model.

6.3.4.3 Data analyses

All results are represented as means ± S.E.M. Statistical comparisons of animal live weights were performed using analysis of variance (Genstat 5, release 4.1,

Lawes Agricultural Trust, 1997). Comparisons between castrates and entires were made using Student's t tests. For the cortisol responses to ACTH and CRH challenges, baseline values were calculated as the average of the two samples collected before the imposition of the challenge. Peak heights were defined as the maximum cortisol concentration observed after administration of the challenge. As remotely collected blood samples are collected continuously over the sampling period (e.g. 10 or 20 min samples), cortisol concentrations for each sample represented an average value for that sampling period. Therefore the area under the cortisol response curve could be calculated by summing the measured concentrations over the sequence of blood samples taken after imposition of the treatments. Comparisons between castrates and entire stags in the estimated clearance rates of cortisol from the circulation following the ACTH and dexamethasone challenges were made by calculating the slope of the log curve over a linear range (40 to 100 min post-infusion for the ACTH challenge and 30 to 105 min for the dexamethasone challenge).

6.4 RESULTS

There were no significant differences in live weight between castrated and entire stags during any stage of the experiment. Animals that were blood sampled lost significantly ($p < 0.05$) more weight (mean net loss 4.2 ± 1.2 and 2.0 ± 0.7 kg for weeks 1 and 2, respectively) than non blood sampled herd mates over the corresponding weeks (mean net loss 0.9 ± 0.7 and 0.1 ± 0.6 kg for weeks 1 and 2, respectively).

Ninety seven percent of samples were collected successfully by remote blood sampling during the 24h baseline sampling periods (Day 2 each week) and 91 % were successfully collected during the ACTH and CRH challenges (Day 4 each week).

6.4.1 Twenty four hour cortisol profiles

Twenty four hour profiles of plasma cortisol concentrations for the 6 entire and 6 castrated stags blood sampled remotely during weeks 1 and 2 are shown in Figure

6.1. Animal E exhibited consistently elevated cortisol concentrations (mean 38.0 ± 1.2 ng/ml) over the 24-h period. Subsequent biochemical analysis of the plasma revealed high levels of creatine kinase (CK), aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) indicating possible liver and muscle damage. Therefore, the data from this animal, though presented in Figure 6.1, were not used in any further analyses.

Mean 24 h concentrations of total plasma cortisol tended to be lower ($p = 0.058$) in the 5 entire animals (3.95 ± 0.75 ng/ml) than the 6 castrated animals (6.22 ± 0.84 ng/ml). All 24 h cortisol profiles were characterised by a pulsatile secretory pattern. The mean cortisol pulse parameter values for the six castrated and five entire stags are shown in Table 6.1. Mean cortisol pulse height, amplitude, and area under the curve were significantly lower ($p < 0.05$) in the entire stags compared with the castrated animals. None of the remaining parameters were significantly different though mean pulse nadir and frequency tended to be lower in the entire animals ($p < 0.063$).

Significant ($p < 0.05$) circadian rhythms in the 24 h cortisol profiles were found in 5 out of 6 castrates and 3 out of 5 entire stags (Figure 6.1). The amplitude of the circadian rhythms did not differ significantly between castrates (2.20 ± 0.53 ng/ml) and entire stags (2.56 ± 1.53 ng/ml), with an overall mean of (2.33 ± 0.54 ng/ml). The time of the day at which the peak in the circadian rhythm occurred also did not differ significantly between castrates (0656 ± 0053 h) and entire stags (0738 ± 0106 h), with an overall mean of (0711 ± 0037 h). The messor of the circadian rhythm was significantly higher ($p = 0.044$) in castrates (6.09 ± 0.85 ng/ml) than entire stags (3.80 ± 0.60 ng/ml).

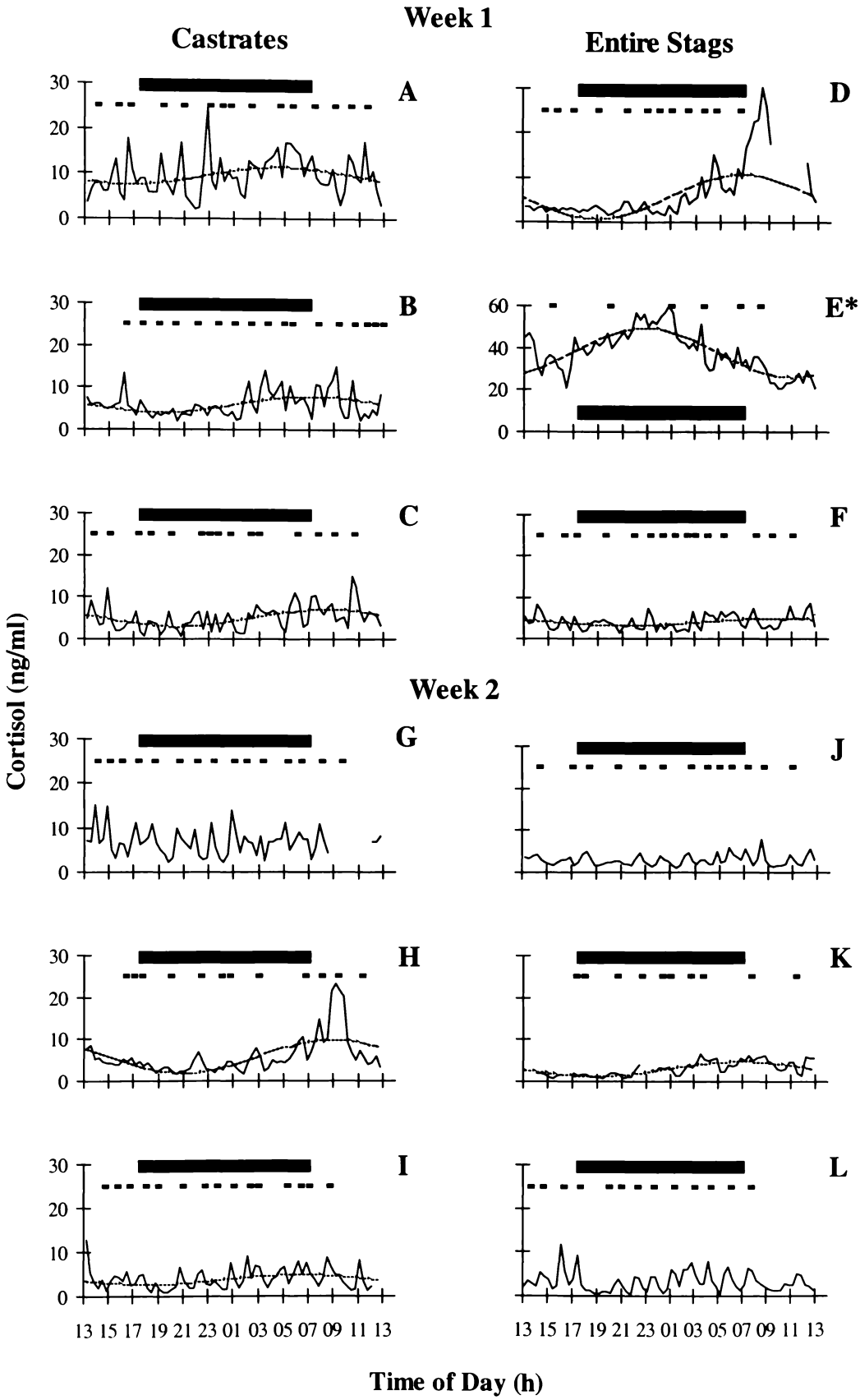


Figure 6.1 Twenty four hour profiles of cortisol concentrations in plasma collected from six castrated red deer stags during week 1 (A, B, C) and week 2 (G,

H, I) and six entire red deer stags during week 1 (D, E, F) and week 2 (J, K, L). The occurrence of cortisol pulses as detected by cluster analysis are shown as points at the top of each graph. Significant circadian rhythms when present are represented by the best fitting sine curve (dashed line) for each data series derived from COSINOR analysis. The black bars at the top of each graph represent the period of darkness. Stag E* exhibited abnormal cortisol concentrations and was excluded from further analyses as subsequent biochemical analysis of the plasma indicated liver and muscle damage.

Pulse:	Entire	Castrate	p value*
Frequency (pulses/h)	0.55 ± 0.03	0.64 ± 0.03	0.063
Interval (min)	96.95 ± 6.12	87.09 ± 4.64	0.182
Height (ng/ml)	5.18 ± 0.49	9.39 ± 1.33	0.015
Amplitude (ng/ml)	3.13 ± 0.48	5.61 ± 0.76	0.018
Nadir (ng/ml)	2.14 ± 0.41	3.77 ± 0.68	0.061
Width (min)	74.61 ± 6.28	64.52 ± 3.98	0.153
Area (ng/ml)	133.51 ± 15.65	226.5 ± 29.78	0.019

Table 6.1 Plasma cortisol pulse parameters (mean ± S.E.M) derived from a cluster analysis of 24 h cortisol profiles collected from six castrated and five entire red deer stags while undisturbed at pasture.*p values derived from students t test.

6.4.2 ACTH and CRH challenge

The mean cortisol concentrations following the ACTH and CRH challenges for both castrated and entire stags are presented in Figure 6.2. Mean baseline plasma cortisol concentrations obtained for the 20 min prior to the challenge with ACTH did not differ significantly ($p = 0.327$) between castrates (5.33 ± 1.09 ng/ml) and entire stags (3.49 ± 1.73 ng/ml).

Peak cortisol concentrations following ACTH challenge were significantly higher ($p < 0.01$) in castrates (53.1 ± 4.13 ng/ml) than entire stags (31.24 ± 4.25 ng/ml). The area under the cortisol response curve followed a similar pattern with greater areas ($p < 0.01$) seen with the castrated animals (338.47 ± 28.3 ng/ml/100 min) than the entires (183.25 ± 41.8 ng/ml/100 min). No significant difference was

observed between castrates and entires in the slope of the exponential curve describing the decline in cortisol was 0.98 ± 0.00 for the castrated and 0.97 ± 0.00 for the entire stags over the period 40 to 100 minutes post-infusion of ACTH.

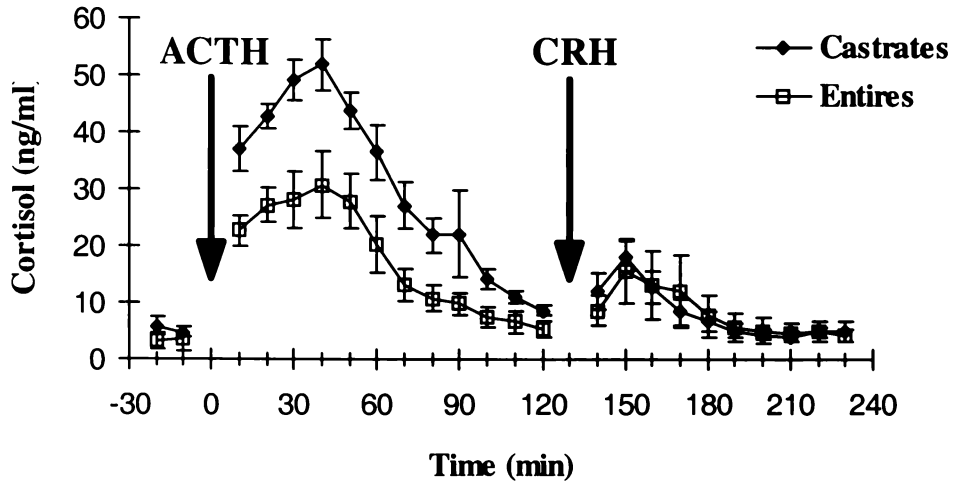


Figure 6.2 Plasma cortisol responses of castrated and entire red deer stags to a remote bolus *i.v.* infusion of ACTH (0.04 iu kg^{-1}) at 0 min, followed by a bolus *i.v.* infusion of CRH (25 ng kg^{-1}) at 130 min.

As with the ACTH challenge, the cortisol concentrations obtained immediately prior to challenge with CRH did not differ significantly ($p = 0.103$) between castrated (8.99 ± 1.45 ng/ml) and entire (5.35 ± 1.56 ng/ml) stags. Peak cortisol responses of 25.12 ± 2.58 ng/ml for castrates and 15.9 ± 5.81 ng/ml for entire stags were not significantly different ($p = 0.181$). The ratio of peak cortisol concentrations (i.e. the mean peak cortisol response of entire stags divided by the mean peak cortisol response of castrates), was similar for both the ACTH challenge (0.588) and the CRH challenge (0.633). The area under the cortisol response curve following CRH challenge was not significantly different ($p = 0.505$) between castrates (105.40 ± 15.56 ng/ml/100min) and entires (82.13 ± 30.53 ng/ml/100 min).

6.4.3 Free cortisol

The proportion of unbound cortisol in plasma in baseline (Low) samples was significantly lower ($p < 0.001$) in castrated (8.93 ± 0.39 %) than in the entire stags (12.91 ± 0.94 %). The derived measures of the concentrations of free plasma cortisol was not significantly different ($p = 0.993$) between castrates (0.41 ± 0.09 ng/ml) and entires (0.41 ± 0.21 ng/ml).

The proportion of unbound cortisol in the (High) were significantly lower ($p < 0.05$) in castrated (17.89 ± 1.03 %) than entire stags (24.01 ± 2.45 %). These values were significantly higher ($p < 0.001$) than in the low samples for exh treatment respectively. There was no significant difference ($p = 0.318$) between castrated and entire stags in the calculated concentration of free plasma cortisol (9.23 ± 0.91 ng/ml and 7.62 ± 1.50 ng/ml, respectively).

6.4.4 Dexamethasone suppression test

The plasma cortisol responses of castrated and entire stags to a dexamethasone suppression test are shown in Figure 6.3. The mean pre-challenge (-30 to 0 min) plasma cortisol concentrations did not differ significantly ($p = 0.199$) between castrates (17.3 ± 3.31 ng/ml) and entires (11.05 ± 3.52 ng/ml). Administering dexamethasone (at time 0) resulted in a decline in plasma cortisol concentrations in both castrated and entire stags. Plasma cortisol concentrations continued to decline to similar ($p = 0.667$) minimum concentrations in both castrates (1.93 ± 0.83 ng/ml) and entire stags (1.55 ± 0.28 ng/ml). The time required to reach these minima were similar ($p = 0.438$) for both castrates (160.0 ± 5.5 min) and entire stags (153.0 ± 8.2 min).

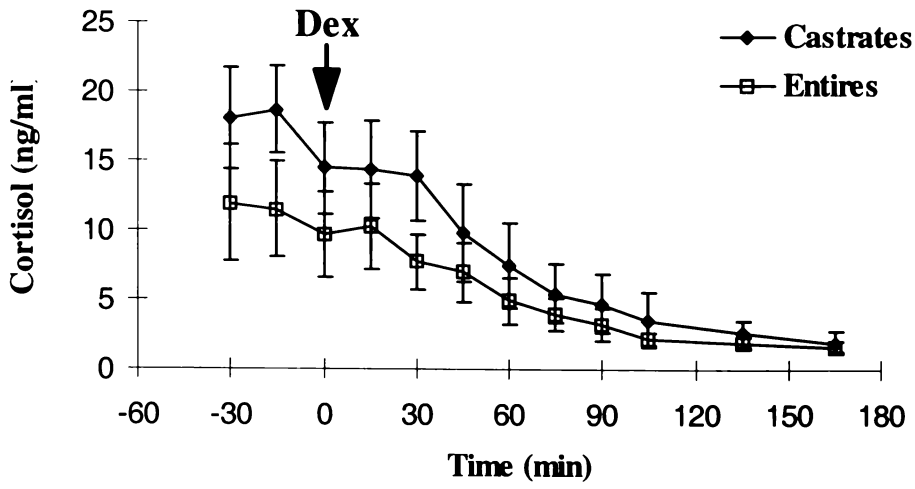


Figure 6.3 Plasma cortisol responses of castrated and entire red deer stags to intravenous administration of dexamethasone sodium phosphate (0.037 mg kg^{-1}) at 0 min.

The slope of the exponential curve describing the decline in cortisol following administration of dexamethasone was calculated for each animal from 30 to 105 minutes post-infusion. No significant differences ($p = 0.167$) were observed between castrates (0.979 ± 0.003) and entire stags (0.984 ± 0.001) in the slope of the curve (over all mean slope 0.981 ± 0.002).

6.5 DISCUSSION

The results of this study clearly indicated differences between castrated and entire stags in several aspects of the activity and functioning of the HPA axis. A number of parameters describing the basal activity of the HPA axis were found to be greater in castrated compared with intact animals. This was reflected in measures of the average height, amplitude and area of cortisol pulses. Castrates also tended ($p < 0.10$) to have higher 24 h mean cortisol concentration as well as cortisol pulse frequencies and interpulse nadirs. This result is similar to that reported for other species where the removal of gonadal steroids by castration of mature males has been shown to result in increased basal concentrations of glucocorticoids, while treatment with testosterone returns basal cortisol concentrations to precastration levels (e.g. rats: Gaskin & Kitay 1971, Handa *et al.* 1994b).

Androgen dependent mechanisms may also play an important role in the marked seasonal rhythm in basal cortisol concentrations exhibited by red deer stags (Ingram *et al.* 1999, Chapter 5). Stags exhibit a well documented annual cycle in reproductive function (Clutton-Brock *et al.* 1982, Suttie *et al.* 1995) with high levels of circulating testosterone during the autumn breeding period (rut) which decline over the winter to very low levels during the period of reproductive quiescence in spring and early summer (Suttie *et al.* 1992). As a consequence, stags are infertile during this period of reproductive quiescence, and can be considered basically as functional castrates.

In the present study, the basal activity of the HPA axis in entire stags was similar to that reported previously from stags during the rut (Chapter 5)(e.g. mean 24 h cortisol concentrations, 4.0 ± 0.8 ng/ml vs. 4.0 ± 1.0 ng/ml, respectively). Cortisol pulse parameters were also similar (e.g. cortisol pulse height 5.2 ± 0.5 ng/ml vs. 5.7 ± 1.3 ng/ml, amplitude 3.1 ± 0.5 ng/ml vs 2.9 ± 0.8 ng/ml, and nadir 2.1 ± 0.4 ng/ml vs 2.8 ± 0.6 ng/ml, respectively). The similarity in basal activity of different groups of stags monitored in different years (1996 and 1997, Chapter 5 and 6, respectively) demonstrates that a low level of HPA axis activity during the rut is a consistent feature of the seasonal physiology of these animals.

The basal HPA axis activity of castrated stags, though greater than intact stags during the rut, was lower than that previously reported for entires monitored during the seasonal nadir in reproductive function (November) (Ingram *et al.* 1999, Chapters 5,7). For example, mean 24 h cortisol concentrations were 6.2 ± 0.8 ng/ml for castrates in the present study and 12.5 ± 1.0 ng/ml and 12.9 ± 1.5 ng/ml for entires in November in Chapters 5 and 7, respectively. Cortisol pulse parameters also exhibited similar differences between castrates in the present study and reproductively quiescent entires during November in Chapters 5 and 7. For example, cortisol pulse height 9.4 ± 1.3 ng/ml vs. 17.1 ± 1.3 ng/ml and 17.8 ± 1.6 ng/ml, amplitude 5.6 ± 0.8 ng/ml vs 9.7 ± 0.8 ng/ml and 9.19 ± 9 ng/ml, and nadir 3.8 ± 0.7 ng/ml vs 7.5 ± 0.6 ng/ml and 8.4 ± 1.2 ng/ml in the present study and Chapters 5 and 7, respectively. This comparison with previously reported data demonstrates a clear difference in basal activity of the HPA axis between castrates during the rut and entire stags during the seasonal period of reproductive

quiescence (spring and early summer). This suggests that the removal of androgen inhibition of HPA axis activity constitutes only a part of the regulatory changes responsible for the higher basal activity observed in entire stags in the other studies during the seasonal period of reproductive quiescence (spring and early summer).

Circadian rhythms in plasma cortisol concentrations have been found previously in red deer using the DracPac remote sampling technique (Ingram *et al.* 1999, Chapters 5 & 7) but have not been identified in other deer species using traditional blood sampling techniques (e.g.white-tailed deer, Bubenik *et al.* 1983; rusa deer, van Mourik & Stelmasiak 1984; and Eld's deer, Monfort *et al.* 1993). It would appear unlikely that red deer are the only cervid species that exhibit circadian rhythms in cortisol. This absence of rhythms in these other deer species would suggest that circadian rhythms in deer are readily attenuated by handling stress. In other species chronic or repeated stress has also been shown to blunt circadian rhythmicity of glucocorticoids by increasing concentrations during the nadir (e.g. rats, Akana *et al.* 1992; horses, Irvine & Alexander 1994). Circadian rhythmicity in the basal activity of the HPA axis was apparent in the present study for both castrated and entire red deer stags. Castrated and entire stags exhibited rhythms of similar amplitudes and timing (acrophase). However, the mesor around which the rhythm fluctuates was significantly higher in the castrates, reflecting the higher level of basal activity in these animals. Circadian rhythms in plasma cortisol concentrations of similar amplitude and acrophase have been previously reported previously for stags during the rut (amplitude, 1.09 ± 0.23 ng/ml, acrophase, 06:42 ± 1:43 h, Ingram *et al.* 1999, Chapter 5).

A difference in adrenal responsiveness to ACTH would appear to account for much of the variance seen between castrates and entire stags in basal activity of the HPA axis. The maximum glucocorticoid response to ACTH challenge in entire stags was only 59 % of that of castrated stags. A similar proportional difference was observed in cortisol pulse parameters derived from basal activity of the HPA axis. For example, in entire stags, cortisol pulse height, amplitude and nadir were only 55 %, 56 % and 57 %, respectively, of castrate values. In red deer stags, the seasonal rhythm in adrenal responsiveness to ACTH involves greater plasma

cortisol responses to ACTH challenge during spring/summer compared with the breeding season and winter months (Cassidy 1996, Suttie *et al.* 1995, Ingram *et al.* 1999, Chapter 5). Adrenal function appears to be inversely correlated to the annual cycle in testosterone concentrations. In the present study, the mean peak cortisol response to ACTH challenge in entire stags (31.2 ± 4.3 ng/ml) were similar to those previously reported for entire red deer stags during the rut (33.7 ± 1.8 ng/ml, Ingram *et al.* 1999, Chapter 5). While peak cortisol responses of castrated stags (53.1 ± 4.1 ng/ml) closely resembled those of entire stags challenged with ACTH during the seasonal nadir in reproductive function (November: 55.8 ± 2.7 ng/ml, Ingram *et al.* 1999, Chapter 5). This would suggest that in stags, much of the seasonal variation in adrenal function is attributable to seasonal changes in the degree of androgen dependent suppression of adrenal function. Androgens may regulate adrenal function by both direct and indirect mechanisms. Androgens have been shown to directly suppress glucocorticoids production by the adrenal cortex by inhibiting the activity of specific steroidogenic enzymes such as 21-hydroxylase and 11β-hydroxylase, which are involved in the synthesis of glucocorticoids (Gaskin & Kitay 1971, Hornsby 1982; Miller 1988).

Androgens may also suppress glucocorticoids production in the adrenal cortex by reducing trophic support from higher levels of the HPA axis. This support is essential for maintenance of adrenocortical function (Simpson & Waterman 1988). For example, lower levels of endogenous ACTH secretion have been reported in intact male compared with castrated rats (Handa *et al.* 1994b) while males of the highly seasonal Soay sheep breed show a marked reduction in endogenous ACTH secretion and adrenal responsiveness to ACTH during the autumn breeding period when testosterone concentrations are maximal (Ssewannyana *et al.* 1990). Though ACTH could not be measured in the present study, an androgen dependent change in trophic support to the adrenal glands is suggested by the tendency for a reduced cortisol pulse frequency in entire stags compared with castrates. A pattern of reduced cortisol pulse frequency has also been described for entire stags during the rut (Ingram *et al.* 1999, Chapter 5). However, a mechanism involving androgens suppressing trophic support to the adrenals is contradicted by reports of adrenal cortex hypertrophy and hyperpalsia

in wild red deer stags (Kapp 1989) and white tailed deer stags (Hoffman & Robinson 1966) during the rut. These data would suggest that trophic support of the adrenal actually increases as the adrenal cortex increases in size in entire animals during the rut, at least in wild populations.

Androgens do not appear to influence the functioning of the pituitary corticotroph in red deer. Both castrates and entire stags exhibited similar responses to CRH challenge. In this respect, red deer are similar to other species, where castration has also been reported to have no affect on pituitary responsiveness to CRH (e.g. rats: Handa *et al.* 1994a). The cortisol response to CRH challenge in both the entire (15.9 ± 5.81 ng/ml) and castrated (25.12 ± 2.58 ng/ml) animals was similar to that measured in entire stags at the time of year when HPA axis activity is at its peak and reproductive function is at its minimum (November, 20.8 ± 1.4 ng/ml, Chapter 7). In contrast to the effects of chronic social stress when anterior pituitary responsiveness to CRH is significantly reduced (Chapter 7), the seasonal rhythm in HPA axis activity in red deer stags appears to involve changes at different levels of the HPA axis other than the anterior pituitary.

Glucocorticoids play a critical role in the negative feedback regulation of the HPA axis. A resistance to the suppressive effects of dexamethasone on glucocorticoid secretion is normally associated with impairment of glucocorticoid negative feedback mechanisms (Sapolsky & Plotsky 1990). No significant differences were observed between castrated and entire stags in the decline in cortisol concentrations following dexamethasone challenge suggesting that glucocorticoid negative feedback mechanisms were functioning in a similar way. This conclusion is supported by studies of corticosteroid receptor populations in the hypothalamus and hippocampus of the rat, which report no differences in MR or GR glucocorticoid receptor concentrations in castrated or entire males (Handa *et al.* 1994b).

Castration of red deer stags in the present study, resulted in an increase in the cortisol binding capacity of their plasma compared with intact animals. Castration of males has been shown to increase CBG concentrations in a number of other species including the rat and guinea pig, while treating castrates with testosterone decreases

the plasma CBG concentration to intact male levels (El Hani *et al.* 1980, Mataradze *et al.* 1992). Androgens are thought to modulate the cortisol binding capacity of plasma via a number of mechanisms. Hepatic synthesis of CBG, the main cortisol binding protein, is suppressed by androgens possibly via neonatal imprinting, which persists throughout adult life (Gala & Westphal 1965, Van Baelen *et al.* 1977). A variety of other factors, which are themselves modulated by androgens (e.g. glucocorticoids, thyroid hormones, growth hormone and the acute phase response) may also alter synthesis of CBG by the liver (Savu *et al.* 1980, Smith & Hammond 1992). In addition, androgens can compete with cortisol for binding sites on corticosteroid binding globulin (CBG) (Siiteri *et al.* 1982, Bradley & Stoddart, 1992) and plasma albumin (Ward *et al.* 1992), resulting in an increased proportion of circulating free cortisol.

Castrated stags also exhibited higher concentrations of total plasma cortisol during both basal activity and peak activity following ACTH stimulation. As the steroid binding capacity of plasma was also higher in castrates the net result was that concentrations of free cortisol were similar in both castrated and entire stags. This was the case during both basal and peak activity. A similar pattern occurred with the seasonal rhythm in HPA axis activity. In entire stags monitored during the seasonal nadir in reproductive function (Chapter 7), the proportion of free cortisol in plasma during basal conditions (9.8 ± 0.6 %) resembled that of castrated (8.9 ± 0.4 %) rather than entire stags during the rut (12.9 ± 0.9 %). In contrast, concentrations of free cortisol (0.32 ± 0.10 ng/ml, Chapter 7) are comparable to those measured in both castrated and entire stags (0.41 ± 0.1 and 0.41 ± 0.2, respectively). Males of other seasonally breeding species exhibit an androgen dependent fall in CBG concentration and hence plasma cortisol binding capacity during the breeding season (McDonald *et al.* 1981, Bradley & Stoddard 1992). It would appear that the maintenance of free cortisol concentrations within a set range by altering plasma steroid binding capacity is important. One possible function of this would be to preserve glucocorticoid bioavailability during the breeding season.

Though only free cortisol is considered biologically active, the maintenance of similar free cortisol concentrations in castrated and entire stags may not necessarily imply a similar functioning of the HPA axis. There is also evidence

that CBG, a member of the serine proteinase inhibitors (serpin) superfamily, promotes the targeted delivery of relatively high concentrations of free glucocorticoids to sites of inflammation (Pemberton *et al.* 1988, Hammond 1997). The CBG-steroid complex may also act as a hormone binding to high affinity specific CBG receptors present on the plasma membrane of a variety of cells (Rosner 1990). Therefore, differences in plasma cortisol binding capacity (or CBG), total cortisol and bound cortisol concentrations (exemplified by differences between castrated and entire stags) not accompanied by changes in free cortisol concentrations still represent a change in HPA axis function.

Plasma cortisol concentrations represent a balance between adrenal secretion and the rate of clearance from circulation. The rate of clearance of cortisol from the circulation following administration of dexamethasone or after the peak adrenal response to ACTH challenge can be used to infer the metabolic clearance rate of cortisol as continued glucocorticoid secretion would normally be suppressed by negative feedback mechanisms in these circumstances. In both cases, castrates and entire stags exhibited similar rates of decline in cortisol concentrations, implying that the cortisol half-life was similar for both groups of deer. A similar situation has been reported previously for cattle, where steers and bulls exhibit similar decay constants for cortisol following an ACTH challenge (Verkerk & Macmillan 1997). This is in contrast to a number of other species where the metabolic clearance rate of glucocorticoids is reduced following castration (e.g. rats: Kitay 1961, guinea pigs: El Hani *et al.* 1980). The higher rate of removal of glucocorticoids from the circulation in mature males of some species represents another androgen dependent mechanism by which lower glucocorticoid concentrations are maintained.

Androgens may also modulate the HPA axis indirectly via changes in behaviour. The period of the rut, with its increased aggression (Suttie1985) and pronounced weight loss (Kay 1979), could be considered a period of increased social and nutritional stress. Prolonged periods of weight loss in humans (over 26 weeks) results in a significant decrease in concentrations of cortisol and cortisol-binding globulin but no change in the concentration of free cortisol (Yanovski *et al.* 1997). In addition, nutritional stress in ruminants is often associated with a metabolic

disorder termed fatty liver syndrome. Red deer stags during the rut in the wild, have a high incidence (80%) of fatty liver syndrome (Kapp *et al.* 1989a) which may impair liver function reducing the synthesis of CBG (Veldman & Meinders 1996) and can directly inhibit steroidogenesis by limiting the availability of cholesteryl esters used in the synthesis of steroid hormones (Morrow *et al.* 1979, Nakagawa *et al.* 1997). Chronic social stress has been reported to reduce plasma cortisol concentrations in female red deer (Goddard *et al.* 1994), yearling red deer (Hanlon *et al.* 1995) and stags in the non-breeding season (Chapter 7) as well as reducing adrenal responsiveness to ACTH (Hanlon *et al.* 1995, Chapter 7), and cortisol binding capacity of plasma (Chapter 7). These changes are all seen in the stag during the rut and may indicate chronic stress. However, chronic social stress in non-breeding stags dramatically reduces the responsiveness of the pituitary to CRH (Chapter 7). This change in pituitary function is not seen in stags during the rut, which may mean that stags do not perceive the rut as a time of chronic stress. It is therefore unlikely that the changes seen in HPA axis activity and function following castration are due to lower levels of stress experienced by castrated animals during the rut.

In contrast to entire stags, the stags castrated after puberty continue to grow new velvet antler throughout the year, as they lack the testosterone required for initiating minerlisation and cessation of growth. It has been suggested that increased cortisol concentrations which accelerate bone loss (Kleerekoper *et al.* 1997) may have a role to play in new antler growth, mineralisation and skeletal bone loss during antler growth in spring (Hillman *et al.* 1973, Suttie *et al.* 1995, Ingram *et al.* 1999, Chapter 5). Whether communication between the processes that control antler growth and development and the HPA axis occurs and whether it is bi-directional have yet to be determined.

The inhibitory influences of the HPA axis upon the reproductive axis are well established in a wide variety of species (reviewed in Rivest & Rivier, 1995). Chronic stress, by inducing prolonged activation of the HPA axis, has the ability to impair reproductive function. The mechanisms by which this occurs are numerous. Glucocorticoids inhibit secretion of LH and GnRH release (Dubey & Plant 1985). CRH can also inhibit GnRH neuronal function via direct synaptic

contacts or indirectly via the circulation (Rivier & Vale 1984, MacLusky *et al.* 1988). Communication between the HPG and HPA axis is also bi-directional, with the HPG axis feeding back upon the HPA axis in a number of species to prevent or inhibit the impairment of reproductive function (Handa *et al.* 1994a). This suppression of HPA axis activity by the HPG axis is also apparent in red deer stags where it may provide an advantage for red deer stags during the breeding season, when inter-male competition for females is intense, to have a reduced activity and responsiveness of the HPA axis as a mechanism in maintaining reproductive competence in the face of increased physical and psychological stress.

In summary, the current data indicate that testicular steroids play a role in modulating the activity and functioning of the HPA axis in red deer stags. Basal activity of the axis is increased in castrated compared with entire stags during the rut. However, the basal activity of castrates during the rut is lower than that previously described for entire stags during the seasonal peak in HPA axis activity (spring and early summer). This would suggest that removal of androgen suppression constitutes only a part of the regulatory changes responsible for the high basal concentrations observed in entire stags during spring early summer. Modulation of HPA axis function by testicular steroids in the rut appears to occur primarily at the level of the adrenal cortex with reduced adrenocortical responsiveness to ACTH in entire stags. The suppressive effect of testicular steroids on adrenal function appears to be the primary mechanism involved in the seasonal rhythm in adrenal function. No effect on pituitary responsiveness to CRH was observed but a tendency for a reduced frequency in pulsatile secretion of cortisol in entire stags during the rut would suggest that the activity of higher centres of the HPA are also under androgen suppression. No evidence was found for any effect of testicular steroids on glucocorticoid receptor mediated negative feedback following dexamethasone challenge. Plasma steroid binding capacity was reduced in intact stags, but unbound cortisol concentrations remained at similar levels for castrates and entires. The change in plasma steroid binding capacity may play an important role in maintaining functionality throughout the seasonal cycle in activity of the HPA axis. This study further highlights the need

for a detailed understanding of the basic physiology of the HPA axis before reliable measures of stress can be determined.

CHAPTER 7 HYPOTHALAMIC-PITUITARY-ADRENAL AXIS AND BEHAVIOURAL RESPONSES TO CHRONIC SOCIAL STRESS IN RED DEER STAGS (*CERVUS ELAPHUS*).

7.1 ABSTRACT

To determine the effect of chronic social stress on behaviour and hypothalamic-pituitary-adrenal axis (HPA axis) activity and function, each of 6 two-year-old red deer stags (Mixed stags) were introduced into an unfamiliar group of 5 stags (6 Base herds, 2-4 yrs old,) for 2 days. Individual Mixed stags were exchanged between Base herds every second day for 12 days. Two groups of 6 two-year-old red deer stags (Unmixed stags) were kept as unhandled controls.

The basal activity of the HPA axis was quantified in the Mixed stags at pasture by measuring plasma cortisol concentrations in blood samples collected over 24 h (72 x 20 min samples) using a remote blood sampling device (DracPac), before the first (Mix 1) and again after the last (Mix 6) mixing event. Adrenocortical responsiveness to ACTH (0.04 iu/kg) and pituitary adrenocortical responsiveness to CRH (0.25 ng/kg) were also measured remotely in the Mixed stags at pasture before Mix 1 and again after Mix 6. The acute effects of mixing were assessed by measuring plasma cortisol concentrations in blood samples collected by remote blood sampler during Mix 1 and Mix 6. In addition, negative feedback regulation

of the HPA axis was assessed after Mix 6 in Mixed stags and in Unmixed stags by measuring plasma cortisol concentrations following *i.v* infusion of dexamethasone (37 μg/kg). Behavioural measures of times spent in maintenance activities lying, grazing, standing, walking and fence pacing as well as the incidences of aggression and distances to nearest neighbour were recorded prior to and during each of the 6 mixing events for Mixed and Base herd stags.

Repeated mixing of individual stags into unfamiliar herds resulted in dramatic weight loss and changes in both behaviour and HPA axis activity and function. Mixed stags were subjected to increased levels of aggression and exhibited reduced grazing and increased pacing of fence lines. They also distanced themselves from the rest of the herd and were less synchronised in their behaviour with the rest of the herd. Changes in HPA axis activity and function in Mixed stags were characterised by a lowering of basal cortisol concentrations (12.7 ± 2.5 to 5.1 ± 1.8 ng/ml), an increase in the proportion of unbound cortisol in Low (9.8 ± 0.6 to 12.2 ± 1.1 %) and High (20.1 ± 1.1 to 26.8 ± 3.3 %) cortisol samples, though free cortisol concentrations did not change. Responsiveness of the adrenal to ACTH declined (e.g. peak cortisol, 42.1 ± 3.2 to 32.6 ±2.9 ng/ml) as did pituitary responsiveness to CRH (peak cortisol, 20.8 ± 1.4 to 11.3 ± 1.9 ng/ml). The increase in plasma cortisol to both the first and final mixing events was similar. Repeated mixing into unfamiliar herds appears stressful to red deer stags and resulted in adaptive changes in both behaviour and aspects of the HPA axis. Changes in HPA axis activity, pituitary and adrenal responsiveness and cortisol binding capacity of plasma may provide mechanisms for ameliorating the deleterious effects of chronic activation of the HPA axis while still allowing the maintainence of baseline HPA axis function during chronic social stress in the stag.

7.2 INTRODUCTION

The adaptation of red deer (*Cervus elaphus*) to intensive farming practices has progressed rapidly since the establishment of deer farming in New Zealand using animals captured from the wild in the 1970's. Despite selective breeding for good

temperament farmed red deer still maintain some of the "flighty" nature characteristic of their wild conspecifics. Previous studies have highlighted the behavioural and physiological reactivity of farmed red deer to both acute (e.g. handling, Carragher *et al* 1997; transport, Waas *et al.* 1997, 1999) and chronic stressors (e.g. indoor housing, Goddard *et al.* 1994, Hanlon *et al.* 1994, Pollard & Littlejohn 1998; social stress, Hanlon *et al* 1995). Exposure to stress has also been linked in red deer to poor reproductive performance (Yerex & Spiers 1987), and an increased susceptibility to infectious diseases (Griffin & Thomson 1998).

Behavioural measures can be used to gauge the level of disturbance for various manipulations/challenges (reviewed in Fraser & Broom, 1990, Broom & Johnson, 1993). In red deer, behavioural stress parameters are of special interest since they may be measured relatively easily and do not expose the animal to additional stressors such as blood sampling. A variety of behavioural parameters have been used to quantify the effect of farm management stresses on animal welfare. These parameters include the time required to resume normal behavioural activity after acute stress (Matthews *et al.* 1990) and perturbations in maintenance activity time budgets (Hanlon *et al.* 1994, 1995, Pollard & Littlejohn 1998). Increased incidences of aggression, and escape/avoidance behaviours such as pacing of paddock boundaries (fence pacing) (Moore *et al* 1985, Pollard *et al.* 1998) as well as measures of animal group dynamics such as spatial relationships (Diverio *et al.* 1996a) have also been used.

Changes in the activity and functioning of the hypothalamic-pituitary-adrenal (HPA axis) are also routinely used to quantify levels of stress in farm animals (Barnett & Hemsworth, 1990, Fraser and Broom, 1990). The use of recently developed remote blood sampling equipment (Ingram *et al.* 1994) has allowed the adrenal response to acute stress to be accurately quantified in red deer (Ingram *et al.* 1994, 1997, 1999, Matthews *et al* 1994, Carragher *et al.* 1997, Waas *et al.* 1997, 1999), without the confounding effects of handling stress associated with more traditional forms of blood sampling. In acute stress situations, increased plasma cortisol responses are associated with more intensive handling procedures (Carragher *et al.* 1997), and may reflect the animal's perception of the severity of the stressor imposed.

The effect of repeated or chronic exposure to a stressor on the HPA axis is, however, not well understood. Various measures of the activity of the HPA axis have been found to be increased, unaffected or depressed by exposure to chronic stressors (Rushen 1991, Jensen *et al.* 1996a). Habituation of the glucocorticoid response to acute or novel stressful stimuli may occur as the stimulus is repeated or becomes more chronic (Rhynes & Ewing 1973). However, other studies have reported glucocorticoid concentrations remaining elevated (Roman-Ponce *et al* 1981) or normalised (Ladewig & Smidt 1989) with continued exposure to chronic stress. In yearling red deer, basal cortisol concentrations are significantly lower following chronic social stress (Hanlon *et al.* 1995) and are lower in subordinate animals (Pollard & Littlejohn 1998). Cortisol concentrations also decline during periods of indoor housing (Goddard *et al.* 1994, 1996, Hanlon *et al.* 1994).

Chronic stress can also lower the cortisol binding capacity of plasma, thereby increasing the proportion of biologically active free cortisol present in circulation (Alexander & Irvine 1998, Blanchard *et al.*1993, Kattesh *et al.* 1980, Spencer *et al.* 1996). The function of this stress-induced fall in binding capacity is unclear, but the resulting increased free glucocorticoid concentrations may have a greater effect on target tissues (Fleshner *et al.* 1995). In addition to its influence on total and free glucocorticoid concentrations, chronic stress has been reported to alter the function of specific elements of the HPA axis, including responsiveness of the adrenal cortex to ACTH (Friend *et al.* 1977, 1979, Ladewig & Smidt 1989) and the pituitary corticotrophs to CRH (Alexander *et al.* 1996, Odio & Brodish 1990). However, quantifying the responsiveness of the adrenal cortex by exogenous ACTH challenge following chronic stress has produced conflicting results. For example, in cattle subjected to heat stress (Roman-Ponce *et al.* 1981), and tethering (Ladewig & Smidt 1989), the adrenal response to ACTH is reduced, whereas, chronic social stress and competition for free stalls caused an enhanced adrenal response in this species (Friend *et al.* 1977, 1979). In red deer, the adrenocortical response to ACTH has been reported to increase relative to baseline concentrations (Hanlon *et al.* 1995), remain unchanged (Hanlon *et al.* 1994) or to decline (Goddard *et al.* 1994, Pollard & Littlejohn 1998) during chronic social stress.

Reduced pituitary responsiveness to CRH is a characteristic of a number of human psychiatric disorders that are indicative of chronic stress. These include depression, panic disorder, anorexia nervosa and post-traumatic stress disorder (Smith *et al.* 1989). In the horse, chronic social stress has also been shown to reduce pituitary responsiveness (ACTH secretion) to an exogenous CRH challenge, and to increase pituitary venous blood concentrations of CRH in resting animals (Alexander *et al.* 1996). The pituitary or pituitary-adrenal response to CRH challenge have not been reported for red deer, however CRH has been shown to be a potent stimulator of ACTH secretion in cultured pituitary corticotrophs from fallow deer (*Dama dama*) (Willard *et al.* 1995).

Impairment of the glucocorticoid receptor-mediated negative feedback of HPA axis activity (glucocorticoid resistance) is another frequently reported effect of chronic stress in rats (Herman *et al.* 1995, Makino *et al.* 1995). In addition, it is also a well established biological marker of human depression (Arana & Mossman 1988), a condition in which chronic stress is implicated. Stress-induced glucocorticoid resistance is believed to result from the hypersecretion of glucocorticoids during stress. This hypersecretion may lead to the down regulation of glucocorticoid receptors, impairing feedback regulation mechanisms and facilitating continued hyperactivity of the HPA axis (Sapolsky & Plotsky 1990). Resistance to the suppressive effect of dexamethasone, a synthetic glucocorticoid, has been shown in rats subjected to inescapable shock (Zhukov 1993) reflecting the impairment of glucocorticoid negative feedback mechanisms. In deer, dexamethasone has been reported to suppress endogenous cortisol secretion when used as a pre-treatment before ACTH challenge (Goddard *et al.* 1994, Smith & Bubenik 1990) but its usefulness in evaluating glucocorticoid receptor-mediated negative feedback has not previously been reported.

The variability in the responses of HPA axis parameters to chronic stress reported in the literature may be due to a number of factors. These include specific species differences, the use of experimental subjects of different sex and reproductive status and differences in experimental protocols, including differing sampling times and methods. The quantitative and qualitative properties of the stressor may also influence responses. For example, many long term stressors do not represent

a constant unvarying state, but a sucession of repeated acute stressors (chronic intermittent stress) which can lead to changes in response with repeated exposure (see Ladewig 2000). In addition, Jensen *et al.* (1996a) proposed that rather than being a permanent state, the response to chronic stress may also change over the time course of exposure to the stressor. These changes are characterised by an initial activation of the HPA axis followed by biochemical adjustments that result in an apparent normalisation or even suppression of the activity in the HPA axis over time (Jensen *et al.* 1996a). Thus, the measured effects of chronic stress on the HPA axis could be influenced by the stage during the process when the axis is evaluated.

The mixing of unfamiliar conspecifics is known to elicit aggression and induce physiological responses typical of stress in a number of social species including rats (Stefanski & Engler 1998), horses (Alexander & Irvine 1998) and red deer (Pollard *et al.* 1993, Hanlon *et al.* 1995, Pollard & Littlejohn 1998). Intense interactions between unfamiliar conspecifics of social species do not usually persist and therefore the acute stress response is generally short-lived (Mormède 1990). However, if individual animals are regularly reintroduced to unfamiliar groups, the result is an extended period of social instability. In red deer, mixing of unfamiliar animals has previously been shown to be stressful both in the short term (Pollard *et al.* 1993, Pollard & Littlejohn 1998) and when mixing is repeated over a longer period of time (Hanlon *et al.* 1995). Mixing of social groups is also thought to contribute to pre-slaughter handling stress in farmed deer (Alexander 1988), and thus may have important economic and animal welfare consequences.

A recent study by Hanlon *et al.* (1995) reported that chronic social stress induced by repeated mixing resulted in a decline in basal cortisol concentrations, an increase in the peak cortisol response to an ACTH challenge and a reduction in cell-mediated immune function in yearling red deer hinds housed indoors. The aim of this study was therefore to investigate the effects of chronic social instability on behaviour and hypothalamic-pituitary-adrenal axis (HPA axis) activity and function in mature red deer stags maintained outdoors. Social instability was induced by repeated mixing of individual stags with unfamiliar groups. To avoid the potential confounding effects of handling and indoor housing

on HPA axis activity, a remote blood sampling device (Ingram *et al.* 1994) was used to obtain blood samples from undisturbed animals at pasture.

7.3 MATERIALS AND METHODS

The following experimental protocol was approved by both the University of Waikato and Ruakura Animal Ethics Committees.

7.3.1 Animals

Eighteen 2-year-old red deer stags, blocked by weight were allocated to 3 groups (n = 6 animals/group) 40 days prior to the start of the experiment (19/11/96). The six animals in one group (Mixed stags, mean liveweight 123.9 ± 5.6 kg) were subsequently subjected to chronic social stress while the members of the other two groups served as Unmixed controls (Unmixed stags, mean liveweight 124.3 ± 2.7 kg). A further 6 groups (n = 5 animals/group) of 2-4-year-old stags (mean liveweight 144.3 ± 3.2 kg) whose composition did not change served as the Base herds into which Mixed individuals were introduced (see procedure). All groups were kept in separate paddocks (0.5 hectare) during the experiment at the Ruakura Agricultural Centre, Hamilton, New Zealand (37°46'S, 175°20'E) with *ad libitum* access to pasture (ryegrass-white clover) and water. Spatial separation between groups was achieved by using alternate paddocks on both sides of a central race.

7.3.2 Procedure and remote blood sampling

Behavioural measures and HPA axis activity and functioning was quantified in the 6 Mixed stags before, during and after a period of chronic social stress. The experiment was conducted from November 19th to December 13th 1996 during the Southern Hemisphere Summer. The experimental procedure is outlined in Figure 7.1.

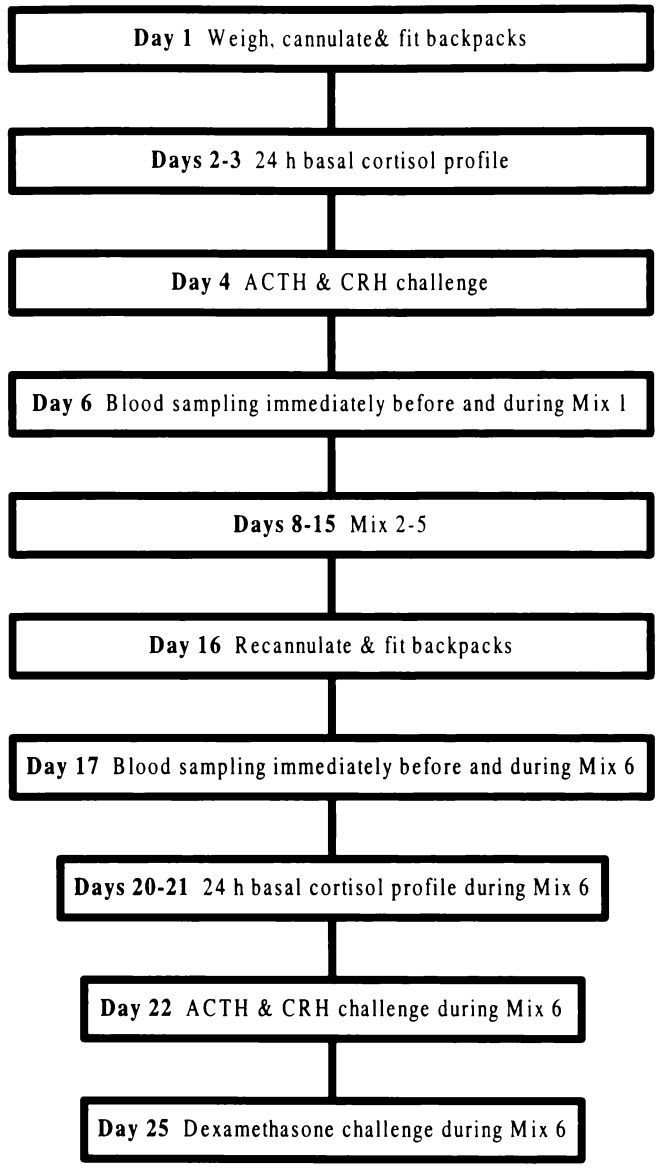


Figure 7.1 A flow diagram outlining the experimental procedure.

7.3.2.1 Pre-mixing 24 h basal cortisol profile

On day one, all animals were weighed and the 6 animals to be remotely blood sampled (Mixed stags) were catheterised and fitted with a canvas and leather backpack as previously described in Chapter 5. Pre-mixing baseline activity of the HPA axis was determined in the Mixed stags prior to being introduced to the unfamiliar Base herds and while still in their original group by blood sampling using a battery powered remote blood sampler (DracPac: ABWRC, AgResearch and

Engineering Development Group, HortResearch, Ruakura Agricultural Centre, Hamilton, N.Z.) over a 24 h period. To do this Mixed stags were penned between 0600 h and 1030 h on Day 2 and each animal was fitted with a DracPac remote blood sampling device. They were then returned to their paddock and allowed to recover from the effects of handling (approx. 3.5 h) before remote blood sample collection commenced at 1300 h. The DracPac was programmed to collect 72 x 20 min samples of blood continuously over 24 h at a rate of 15 ml per hour. The procedure and equipment were as previously described in Chapter 5. At the end of this 24 h collection period (1300 h Day 3), the animals were returned to the yards and the DracPac and blood samples were removed from the backpack.

7.3.2.2 Pre-mixing ACTH and CRH challenge

The glucocorticoid responses to remotely administered ACTH and CRH challenges were quantified on Day 4. Mixed stags (still in their original group) were again penned between 0600 and 1030 h and a DracPac fitted. Animals were then returned to their paddock for a period of recovery (approx. 3.5 h) before remote blood sample collection commenced at 1300 h. Two 10 min baseline samples (30 ml/h) were then automatically collected, before 2 ml of physiological saline containing ACTH $_{1\text{-}24}$ (0.04 IU kg^{-1} live weight; Synacthen; Ciba NZ, Auckland, NZ) was infused into the jugular vein over 5 min starting at 1320 h (time 0, Figure 7.3). Fresh blood was then drawn from the animal through the blood lines to a waste tube for 5 min to clear the blood lines of saline. Following the ACTH infusion and flushing of blood lines (total time 10 min) twelve 10 min blood samples (30 ml/h) were then collected over 120 min. Two hours after the ACTH challenge at 1530 h, (time 0, Figure 7.4), 2 ml of physiological saline containing, Bovine CRH (25 ng kg^{-1} live weight, Sigma), Bovine Serum Albumin (10 mg ml^{-1}, Sigma) and L-Ascorbic Acid (1 mg ml^{-1}, Sigma) was infused over 5 min into the jugular vein of the animals followed by flushing of the lines as per the ACTH challenge. Ten 10 min blood samples (30 ml/h) were then collected before returning the animals to the yards where the blood samples, sampling equipment and harnesses were removed.

7.3.2.3 Mixing

Animals were given a day to recover (Day 5) before being returned to the handling facility on the morning of Day 6. The Mixed stags were penned and fitted with the remote blood sampling equipment before been returned to their paddock (still in original group), for a period of recovery before remote blood sampling commenced at 1320 h. Two 20 min baseline samples (15 ml/h) were then automatically collected before animals were again returned to the handling facility (1400 h) where each of the Mixed group stags were drafted out of the group and introduced into one of the six unfamiliar Base herds. The Base herds, which now included one Mixed group stag in each were then returned to their respective paddocks (approx. 1500 h). The mixing procedure from yarding to returning animals to their respective paddocks took on average 59 ± 5 min. Remote blood samples were collected every 20 min throughout the drafting and mixing procedure and while the animals were at pasture until 2000 h. Behavioural recordings (detailed below) were also made on the groups at pasture for the first 6 h after the time of release from the handling facility and for a 3 h period the following day. Following collection of the last blood sample animals were returned to the handling facility where the blood samples and all equipment including backpacks and catheters were removed.

Every 48 h Mixed stags were subjected to further mixing events (mixes 2 to 5, Days 8-15) without blood sampling. This involved returning the 6 Base herds which included a Mixed group stag in each, to the handling facility at 1400 h. All animals were penned and each of the Mixed group stags were drafted out of their current Base herd and introduced into unfamiliar ones. Animals were then returned to their respective paddocks (approx. 1500 h) where their behaviour was recorded for the following 6 h and for a 3 h period the following day (see section 7.3.3 for details).

Blood samples were collected for the final mixing event (mix 6, Day 17) after recannulating the previous day (Day 16). The procedure was the same as that for the first mixing event (mix 1, Day 6) with the exception that each Mixed stag was part of its Mix 5 Base herd and not part of its original group during the baseline period (1320 to 1400 h) prior to mixing.

7.3.2.4 Post-mixing 24 h basal cortisol profile

The effect of chronic social stress on basal activity of the HPA axis of Mixed stags was determined on Days 20-21, three days after introduction into their sixth unfamiliar Base herd group and while they remained part of this group (Post-mixing 24 h basal cortisol profile). Basal activity was quantified over a 24 h period using a procedure similar to that described for the pre-mixing 24 h basal cortisol profile (Day 2-3). The exception being that each Mixed stag was part of its Mix 6 Base herd and not part of its original group during the sampling.

7.3.2.5 Post-mixing ACTH and CRH challenge

On Day 22 a second ACTH and CRH challenge (post-mixing ACTH and post-mixing CRH) was administered to Mixed stags (five days after introduction into their sixth unfamiliar Base herd group). A procedure similar to that described for the pre mixing ACTH and CRH challenges given on Day 4 was used, though again the exception was that Mixed stags were kept as part of their Mix 6 Base herd and not part of its original group during the sampling.

7.3.2.6 Dexamethasone suppression test

The effect of chronic social stress on glucocorticoid mediated negative feedback regulation of the HPA axis was assessed by administering to Mixed stags and Unmixed controls a dexamethasone suppression test. In preparation for this test all animals were weighed (Day 23) and the 2 groups of Unmixed controls (n = 6 per group) were cannulated using a similar procedure to that described for the Mixed stags. On Day 25 the Mixed stags were removed from their respective Base herds and together with Unmixed controls were returned to the handling facility (approx. 1000 h) and each group held separately in one of three indoor pens (2.4 x 2.4 m). Manual blood sampling of the catheterised animals was used as the number of animals required precluded the use of remote blood samplers. In addition, handling raises cortisol and any effects of suppression may be more obvious. Manual sampling started at 1200 h with five baseline blood samples (10 ml) being collected at 30 min intervals. Immediately following collection of the fifth sample (1400 h), the group of Mixed stags and one group of Unmixed controls received

dexamethasone sodium phosphate (37 μg kg^{-1} live weight Dexone-5, Bomac Laboratories Ltd, Auckland, New Zealand) via the catheter which was then flushed with saline. The second group of Unmixed controls received 2 ml of physiological saline. Following administration of dexamethasone a further 10 blood samples (10 ml) at 30 min intervals were collected. After the final sample was collected at 1900 h, all animals had their catheters removed and were administered (*i.m.*) a prophylactic dose of antibiotic (Tripen LA, Ethical Agents Ltd., Auckland, N.Z) before returning them to their respective paddocks.

7.3.2.7 Plasma cortisol analyses

All blood samples were centrifuged (1200 g for 15 min) after removal from the backpacks (remotely collected samples) or immediately after manual collection, and the plasma stored at -20°C until assayed for cortisol. Plasma cortisol concentrations were determined in duplicate, after extraction in ethyl acetate, by an ^{125}I radioimmunoassay method with polyethylene glycol separation (Ingram *et al.* 1994). A standard curve, using charcoal stripped deer plasma, was constructed and used to determine the concentration of cortisol in individual plasma samples. The cross-reactivity of the antiserum with 11 deoxycortisol was 5.7% and with cortisone was 1.2%. The inter and intra-assay co-efficient of variations for spiked deer plasma controls of known low (5 ng/ml), medium (20 ng/ml) and high (50 ng/ml) cortisol concentrations were 9.0 % and 13.6 % (low), 5.7 % and 10 % (medium), 3.0 % and 12.8 % (high), respectively. Assay sensitivity was 0.4 ± 0.2 ng/ml.

7.3.2.8 Free cortisol analyses

The unbound (free) cortisol fraction in plasma was determined before and after chronic social stress using samples with low (basal) and high (peak) cortisol concentrations. The Low samples were from the baseline period prior to ACTH challenge and high samples corresponded to the period of peak cortisol responses following ACTH challenge. The unbound cortisol fraction in plasma was quantified in undiluted red deer plasma by the centrifugal ultrafiltration dialysis method of Hammond *et al.* (1980) which is described in Chapter 6. The absolute concentration of free cortisol (ng/ml) was derived by multiplying the proportion of free cortisol in

the sample by the total cortisol concentration obtained from the plasma cortisol assay. Inter and intra-assay co-efficient of variations for the analysis of free cortisol obtained from an assay control of pooled red deer plasma samples were 19.9 and 23.3 % respectively.

7.3.3 Behavioural recording

Behavioural activity was recorded during the first 6 hours of each mixing event (1500 h to 2100 h) and at the same time of day during remote blood sampling for the pre-mixing and post mixing 24 h basal cortisol profiles. An additional 3 h period of behavioural recording was made in the evening of the second day of each mixing event (1800 h to 2100 h) to coincide with the diurnal increase in behavioural activity. Selected maintenance behaviours, agonistic interactions received or initiated and nearest neighbour distances were recorded for each Mixed stag. In addition, during mixing events one member of each Base herd group (Base Herd Control) was observed. Recordings were made by observers concealed in a observation tower located at least 50 m from any of the paddocks containing deer. During the pre-mixing 24 h basal cortisol profile (Day 2) only the single group containing all the Mixed stags was observed. During each mixing event and during the post-mixing 24 h basal cortisol profile (day 20) all 6 Base herds containing a single Mixed stag were observed.

7.3.3.1 Recording of maintenance activity

Maintenance activities and nearest neighbour distance were recorded by scanning the groups every 10 minutes with the aid of binoculars (Pentax 7x50 PCF, Asahi Optical Co. Ltd., Japan). Maintenance activities were categorised as standing (standing stationary), grazing (whilst either standing or walking slowly), lying (sternal or lateral recumbency) and walking. Fence pacing, defined as walking parallel to the direction of a fence while within 1 m of that fence was also recorded. The distance to the nearest neighbour for the Mixed and the nominated Base Herd Control individuals in each group was estimated and categorised as either within 1 body length (1), between 1 and 3 body lengths (2), over 3 body lengths (3) or at least the

width of the paddock or ½ its length (4). Paddock dimensions were approximatelv 50 x 100 m.

7.3.3.2 Recording of agonistic behaviours

The number of agonistic behaviours between Mixed and Base herd members was recorded using the technique of continuous behavioural sampling every half hour for a 5 min period per group. During each mixing event and during the post-mixing 24 h basal cortisol profile (day 20) 2 Base herds were observed at a time using two trained observers. Initial interactions in the paddock following the group's return from the handling facility after mixing were also recorded on to videotape. Only agonistic interactions directed at or initiated by Mixed stags were recorded. Agonistic interactions were categorised into low intensity and high intensity threats and were defined as follows (adapted from Appleby, 1983):

Low intensity threats: Displacement by walking towards
Prevention of approach by lifting head
Laying ears back
Antler threat: nodding or head-tossing
Chin threat: lifting the chin
Foot threat: stiff kicking with the foreleg towards opponent
Stalking: a walking chase of the opponent

High intensity threats: Rushing at, or chasing opponent
Kicking with one or both legs
Biting the opponent
Rearing up and slapping with the forefeet (boxing)

7.3.3.3 Temporal synchronisation of behaviour

The temporal synchronisation of lying, grazing, walking and standing behaviour between Mixed stags or Base Herd Controls and the remainder of the Base herd (n = 4) was investigated by calculating the proportion of the 4 deer in the Base herd group engaged in the same behavioural activity as either the Mixed or Base Herd Control focal animal. A similar measure was calculated for the Mixed stags

during the pre-mixing 24 h profile by comparing each animals behaviour with that of members of the pre mixing group. The values used in the statistical analyses were obtained by calculating the averages per hour of the proportion of synchronisation for each 10 min scan.

7.3.4 Statistical analysis

7.3.4.1 Pulse detection

Discrete pulses in cortisol secretion were quantified using cluster analysis (Veldhuis & Johnson, 1986), a statistically based peak detection algorithm (see Chapter 5 for details). A cluster size of one point for the nadir and one point for a peak was used along with a constant 8.5 % coefficient of variation, a minimum peak size of 0.45 ng/ml and a pooled t statistic of 1 to limit the false peak detection rate to approximately 10 % (Veldhuis & Johnson, 1986). The pulse parameters measured for each 24 h profile were pulse frequency, pulse height, pulse amplitude, nadir and peak width.

7.3.4.2 Circadian rhythm detection

Circadian rhythms in plasma cortisol concentrations were detected using a single cosinor model developed for Microsoft Excel (Microsoft Corp., USA) by Bourdon *et al.* (1995) (see Chapter 5 for details). The mean which the circadian rhythm fluctuates around (mesor), the amplitude of the circadian rhythm and the time of day at which the circadian rhythm was at its peak (acrophase) were obtained using this model.

7.3.4.3 Statistical analyses

Plasma cortisol concentrations and behavioural measurements are represented as means ± S.E.M. Statistical comparisons between groups of animal live weights and cortisol responses to dexamethasone suppression were performed using analysis of variance (Genstat 5, release 4.1, Lawes Agricultural Trust, 1997). Within subject statistical comparisons of changes in animal live weights and cortisol parameters pre and post mixing and the cortisol response to the first and final mixing events were performed using paired two-sample student's t-test. For

the cortisol responses to ACTH and CRH challenges, and the first and final mixing events, baseline values were calculated as the average of the two samples collected before the imposition of the challenge. Peak heights were defined as the maximum cortisol concentration observed after administration of the challenge. As remotely collected blood samples are collected continuously over the sampling period (e.g. 10 or 20 min samples), cortisol concentrations for each sample represented an average value for that sampling period. Therefore the area under the cortisol response curve could be calculated by summing the measured concentrations observed after imposition of the treatments.

Behavioural responses to mixing were compared in two ways: within the first 6 h observation period of each mixing event averaged across all 6 mixing events or between mixing events by averaging across all 6 hours for each mixing event. Statistical comparisons of behavioural responses were performed using analysis of variance for repeated measurements (Genstat 5, release 4.1, Lawes Agricultural Trust, 1997). A blocking structure was used to separate the between and within animal variation. When significant ($p < 0.05$) trends were present, comparisons between mixing events or between average hourly values were made using Student's t test.

7.4 RESULTS

The percentage of samples collected successfully using the DracPac technique was 99 % and 60 % for the pre-mixing and post-mixing 24h basal cortisol sampling, respectively. For the ACTH and CRH challenges, 97 % and 83 % of samples were collected successfully during the pre-mixing challenges and 100 % and 80 % for the post-mixing challenges, respectively. Ninety eight percent of samples were successfully collected during the first mixing event and 86 % during the 6^{th} mixing. One animal (stag E) developed a pressure sore under the back pack during the pre-mixing ACTH and CRH challenge and was excluded from further remote blood sampling. As a likely result of this injury basal cortisol concentrations during the pre-mixing ACTH and CRH challenges were

abnormally elevated for this animal and therefore its data were excluded from further analyses.

7.4.1 Live weight changes

On average stags comprising the Base herds were heavier ($p < 0.01$) than the Mixed group stags (144.3 ± 3.2 and 123.9 ± 5.6 kg, respectively). However, there was no significant difference in initial live weights between Mixed stags and Unmixed control stags (124.3 ± 2.7 kg). By the end of the experiment (23 days) Mixed stags had lost a significant ($p < 0.01$) amount of weight (10.1 ± 1.8 kg) whereas, live weights of Unmixed animals which were left undisturbed until challenged with dexamethasone, increased significantly ($p < 0.001$) by 5.0 ± 0.8 kg. Base herd stags, which were subjected to repeated handling and changes in group composition only gained an average of 2.3 ± 0.8 kg over the same period. By the end of the experiment Mixed stags were significantly ($p < 0.05$) lighter than Unmixed controls and the Base herd stags remained significantly heavier than both Mixed and Unmixed animals ($p < 0.01$ and $p < 0.01$, respectively).

7.4.2 Twenty four hour cortisol profiles

Complete 24 h profiles of plasma cortisol concentrations were obtained from all six Mixed stags during the pre-mixing 24 h basal cortisol profile but from 3 animals only during the post-mixing 24 h basal cortisol profile. The decline in numbers was due to equipment failure (n = 2 stags) and the removal of stag E from further remote blood sampling (see above). Statistical comparisons were made between pre and post mixing 24 h cortisol profiles for these 3 animals only. Given that the number of animals for comparison is low (n=3), the statistical significance of any differences should be treated as indicative only. The 24 h profiles of plasma cortisol concentrations successfully obtained from Mixed stags before and after repeated mixing are shown in Figure 7.2. Visual inspection of the data, supported by statistical comparison using paired Student's t-test, suggests that mean 24 h concentrations of plasma cortisol declined ($p = 0.037$, paired values only) from an average of 12.7 ± 2.5 ng/ml to 5.1 ± 1.8 ng/ml following repeated mixing. The average for all 6 stags prior to mixing was 12.9 ± 1.5 ng/ml.

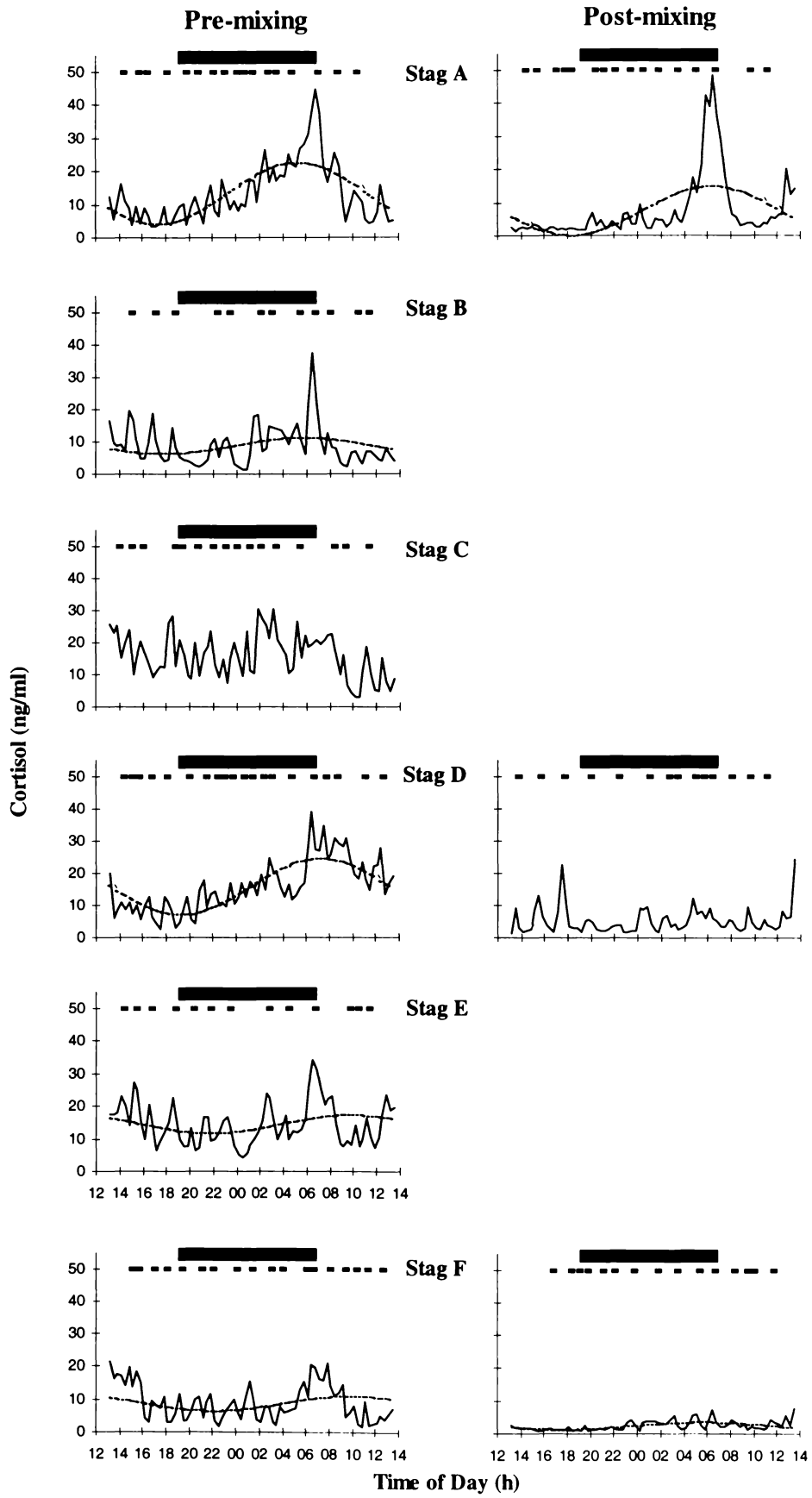

Pre-mixing
Post-mixing
Stag A
Stag B
Stag C
Stag D
Stag E
Stag F
Cortisol (ng/ml)
0
10
20
30
40
50
12 14 16 18 20 22 00 02 04 06 08 10 12 14
Time of Day (h)

Figure 7.2 Twenty four hour profiles of cortisol concentrations in plasma collected from Mixed stags prior to (Pre-mixing) and following the final mixing into novel Base herds (Post-mixing). The occurrence of cortisol pulses as detected by cluster analysis are shown as points at the top of each graph. Significant circadian rhythms are represented by the best fitting sine curve (dashed line) for each data series derived from COSINOR analysis. The black bars at the top of each graph represent the period of darkness

All 24 h cortisol profiles were characterised by a pulsatile secretory pattern and were described by the following parameters; mean pulse frequency, mean pulse interval, mean pulse height, mean pulse amplitude, mean pulse width and mean nadir. The mean values for the 24 h cortisol profiles obtained from 6 stags prior to mixing and the 3 stags following mixing are shown in Table 7.1.

	Pre-mixing (n = 6)	Pre-mixing (n = 3)	Post-mixing (n = 3)	p value
24 h Mean	12.9 ± 1.5	12.7 ± 2.5	5.1 ± 1.8	0.037
Pulse:				
Frequency (pulses/h)	0.67 ± 0.06	0.76 ± 0.05	0.63 ± 0.03	0.149
Interval (min)	87.2 ± 7.6	74.0 ± 2.2	87.5 ± 6.7	0.203
Height (ng/ml)	17.8 ± 1.6	16.3 ± 2.3	6.9 ± 2.1	0.001
Amplitude (ng/ml)	9.2 ± 0.9	7.6 ± 0.8	4.4 ± 1.5	0.062
Nadir (ng/ml)	8.4 ± 1.2	8.7 ± 1.9	2.5 ± 0.6	0.034
Width (min)	66.1 ± 7.3	53.3 ± 2.8	64.8 ± 5.9	0.233

Table 7.1 Baseline plasma cortisol pulse parameters (mean ± S.E.M.) derived from cluster analysis of 24 h cortisol profiles collected prior to (Pre-mixing) (n = 6) and following repeated mixing (Post-mixing) (n = 3). The mean values obtained pre mixing for the 3 post-mixing animals are also given. Statistical significance (p values) derived from paired students t-test is also shown.

Mean cortisol pulse height and nadir were significantly ($p < 0.05$) lower after repeated mixing with the difference in mean pulse amplitude after mixing approaching significance ($p = 0.062$). Values for cortisol pulse frequency, pulse interval and pulse width were not significantly different prior to and following mixing. Cortisol pulses occurred on average every 92 min (0.65 ± 0.04 pulses/h).

Significant ($p < 0.05$) circadian rhythms were found in 5 of the 6 pre-mixing and 2 of the 3 post-mixing 24 h cortisol profiles. The mean amplitude and acrophase of the circadian rhythm did not differ significantly between pre-mixing (Amp. 5.1 ± 1.8 ng/ml, Acro. 07:33 ± 01:06 h:min) and post-mixing (Amp. 4.2 ± 4.4 ng/ml, Acro. 05:56 ± 00:28 h:min) 24 h profiles, with an overall mean amplitude of 4.9 ± 1.4 ng/ml and the timing of the acrophase at 07:05 ± 0:48 h. However the mesor, the mean which the circadian rhythm fluctuates around was significantly higher ($p < 0.05$) pre-mixing (12.4 ± 1.6 ng/ml) compared to post-mixing (5.1 ± 3.5 ng/ml).

7.4.3 ACTH challenge

The mean plasma cortisol responses of 5 stags to remote bolus infusion of ACTH before (pre-mixing) and after repeated mixing (post-mixing) are shown in Figure 7.3. Pre-challenge plasma cortisol concentrations were significantly higher ($p < 0.05$) in stags before repeated mixing (3.2 ± 0.9 ng/ml) than after it (1.1 ± 0.2 ng/ml). Peak cortisol concentrations following the ACTH challenge were significantly higher ($p < 0.01$) in animals prior to the repeated mixing stress (42.1 ± 3.2 ng/ml) than in the same stags after mixing (32.6 ± 2.9 ng/ml). Peak cortisol concentrations remained significantly higher ($p < 0.05$) during the pre-mixing ACTH challenge when values were corrected for baseline differences (pre-mix 38.9 ± 3.6, post-mix 31.5 ± 3.0). The area under the cortisol response curve following ACTH challenge was greater ($p = 0.039$) prior to mixing (233.8 ± 32.8 ng/ml/120 min) than following mixing (177.8 ± 28.4 ng/ml/120 min). No significant difference was observed between pre- and post-mixing in the timing of the peak cortisol response to ACTH challenge. The average time to reach the peak in cortisol was 35 ± 3 min.

The slope of the exponential curve describing the decline in cortisol following the peak over the period 40 to 100 minutes post infusion was used to quantify differences between stags prior to and following repeated mixing stress on the rate of metabolism of cortisol. No significant differences were observed between pre- and post-mixing declines in cortisol concentrations following ACTH challenge (over all mean slope 0.970 ± 0.002).

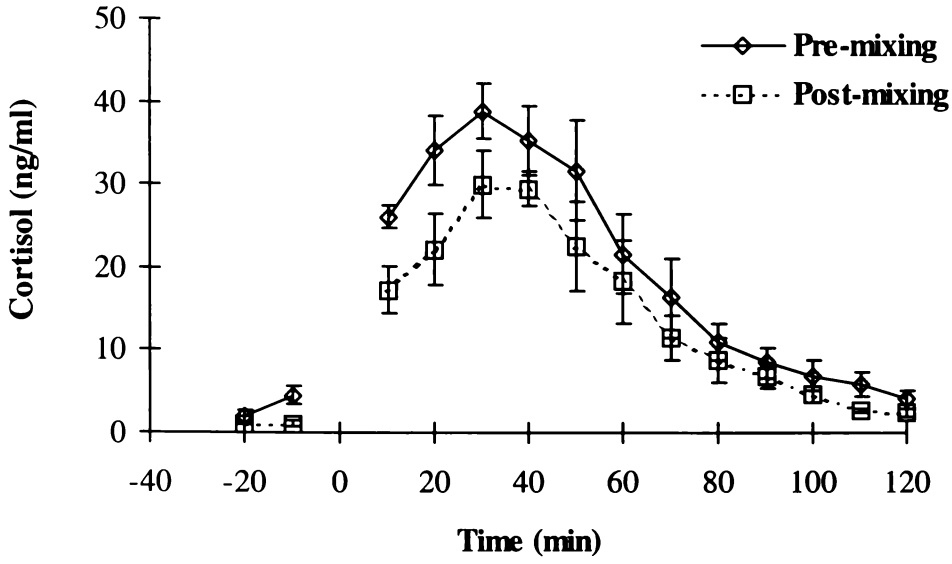


Figure 7.3 Mean plasma cortisol response to remote bolus infusion of 0.04 iu/kg ACTH (time 0 min) prior to (pre-mixing) and following (post-mixing) repeated mixing.

7.4.4 CRH Challenge

The mean plasma cortisol responses of the Mixed stags to remote bolus infusion of CRH prior to (pre-mixing) and following repeated mixing (post-mixing) are shown in Figure 7.4. Pre-challenge baseline plasma cortisol concentrations tended to be lower ($p = 0.072$) in stags after the repeated mixing stress (2.7 ± 0.4 ng/ml) than before it (5.0 ± 1.3 ng/ml). Peak cortisol concentrations were significantly higher ($p < 0.01$) in animals prior to mixing (20.8 ± 1.4 ng/ml) than in the same animals following repeated mixing stress (11.3 ± 1.9 ng/ml). Peak cortisol concentrations were still significantly higher ($p < 0.05$) during the pre-mixing CRH challenge when values were corrected for baseline differences (pre-mix 15.8 ± 2.2, post-mix 8.6 ± 2.1). The cortisol response to CRH challenge in stags prior to mixing exhibited a biphasic response while the same animals after repeated mixing stress exhibited only the first peak. The timing of the cortisol peak to CRH challenge tended to be later ($p = 0.051$) prior to mixing (54 ± 3 min) than following repeated mixing (30 ± 11 min).

The area under the cortisol response curve following CRH challenge was significantly greater ($p < 0.01$) in animals prior to mixing (117.2 ± 14.3 ng/ml/100

min) than in the same animals following repeated mixing (47.0 ± 6.4 ng/ml/100min).

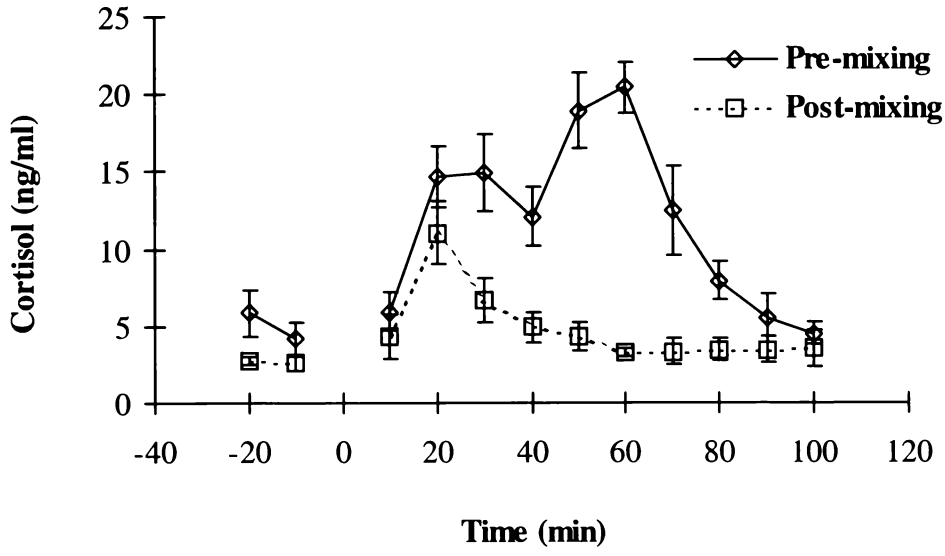


Figure 7.4 Mean plasma cortisol response to remote bolus infusion of 25 ng/kg bovine CRH (time 0 min) prior to (Pre-mixing) and following (Post-mixing) repeated mixing.

7.4.5 Unbound cortisol

The proportion of unbound cortisol was significantly lower ($p < 0.05$) in Low cortisol samples prior to mixing (9.8 ± 0.6 %) than following repeated mixing (12.2 ± 1.1 %). However, total cortisol concentrations in the same samples were significantly higher ($p < 0.05$) before mixing (3.2 ± 0.9 ng/ml) than after repeated mixing (2.0 ± 0.5 ng/ml). Free cortisol concentrations, obtained by multiplying the proportion unbound by the total cortisol concentration, were not significantly different ($p = 0.157$) between animals prior to (0.32 ± 0.10 ng/ml) and following (0.24 ± 0.06 ng/ml) mixing stress.

The proportion of unbound cortisol tended to be lower ($p = 0.059$) in High cortisol samples prior to mixing (20.1 ± 1.1 %) than following repeated mixing (26.8 ± 3.3 %). Total cortisol concentrations in the same samples were significantly higher ($p < 0.01$) before mixing (42.1 ± 3.2 ng/ml) than after repeated mixing (32.6 ± 2.9 ng/ml). There was no significant difference ($p = 0.671$) in the

concentration of unbound cortisol prior to (8.48 ± 0.79 ng/ml) and following mixing (8.96 ± 1.82 ng/ml).

7.4.6 HPA axis response to mixing

The plasma cortisol profiles of the Mixed stags (n = 5) during the first and final mixing are shown in Figure 7.5. The average baseline cortisol concentration obtained prior to mix 1 and mix 6 was numerically greater in mix 1 (11.1 ± 3.3 ng/ml) than mix 6 (3.8 ± 0.8 ng/ml) though this difference was not significant ($p = 0.091$). The peak cortisol concentration was similar ($p > 0.05$) following mix 1 (54.3 ± 5.5 ng/ml) and mix 6 (48.3 ± 3.3 ng/ml) as was the increase in plasma cortisol concentrations relative to baseline (mix 1, 43.2 ± 7.7, mix 6, 44.5 ± 2.7 ng/ml, $p > 0.05$). The area under the curve for the period 0 to 350 min was similar during the first and final mixing (359.2 ± 51.9 and 240.5 ± 31.5 ng/ml/350 min, respectively). Plasma cortisol concentrations of the Mixed stags had returned to values not significantly different from pre-mixing concentrations by 110 min following the start of mixing for both the first and final mixing event (i.e. 50 min after returning to the paddock).

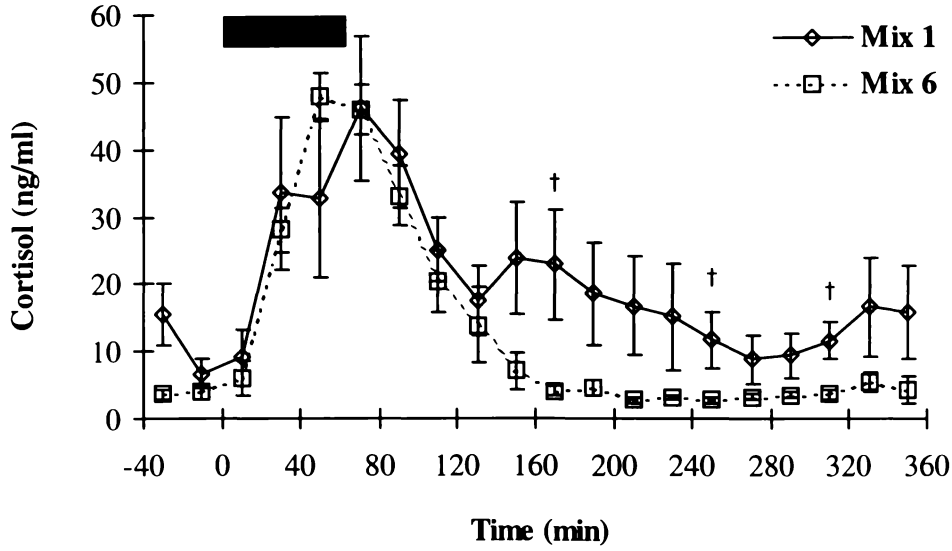


Figure 7.5 The mean plasma cortisol response of 5 stags to the first (Mix 1) and final (Mix 6) mixing event. The black bar at the top of the graph represents the period of time in the yarding facility. † represents time points with a ($p < 0.10$) significant differences between mixes.

7.4.7 Dexamethasone

The plasma cortisol response of the 6 Mixed stags and 6 Unmixed stags to dexamethasone challenge and 6 Unmixed stags to infusion of saline while kept in indoor pens is shown in Figure 7.6. The baseline plasma cortisol concentration was calculated as the average of the samples collected prior to dexamethasone challenge (-120 to 0 min). The baseline values were significantly lower ($p < 0.05$) for Mixed stags (17.1 ± 3.0 ng/ml) than Unmixed stags which were to receive dexamethasone (42.0 ± 7.1 ng/ml) or saline (39.9 ± 8.6 ng/ml).

Plasma cortisol concentrations at time zero for Mixed stags (14.3 ± 2.4 ng/ml) were significantly lower ($p < 0.05$) than Unmixed stags receiving dexamethasone (32.1 ± 6.9 ng/ml) and not different from those from Unmixed stags receiving saline (22.9 ± 4.4 ng/ml). After time zero cortisol concentrations remained unchanged for Unmixed saline controls and declined for the Mixed and Unmixed stags. Cortisol concentrations reached similar ($p = 0.693$) minimum concentrations of 1.8 ± 0.3 and 2.1 ± 0.7 ng/ml, for Mixed and Unmixed stags receiving dexamethasone, respectively. A similar ($p = 0.549$) amount of time was required to reach the minimum cortisol concentration for both the Mixed (230 ± 26 min) and Unmixed stags (250 ± 23 min) after receiving dexamethasone. The slope of the exponential curve describing the decline in cortisol following administration of dexamethasone was calculated for each animal over the period 30 to 120 minutes post infusion. The slope of the curve was significantly ($p < 0.01$) steeper in the Unmixed (0.978 ± 0.002) group given dexamethasone than the Mixed stags (0.986 ± 0.002). Both dexamethasone treated Mixed and Unmixed stags had significantly ($p < 0.001$) steeper curves than Unmixed stags administered saline (1.000 ± 0.002).

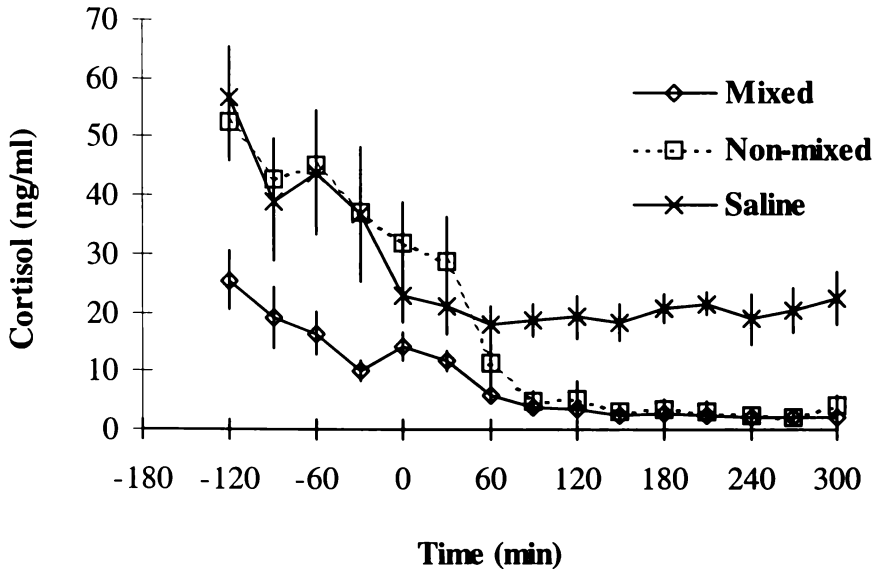


Figure 7.6 Plasma cortisol responses of the Mixed (Mixed) and Unmixed (Non-mixed) red deer stags to intravenous administration of 0.037 mg/kg of dexamethasone sodium phosphate or non-mixed stags to saline (Saline) given at time zero.

7.4.8 Behavioural response

7.4.8.1 Aggression

The frequency of low and high intensity agonistic behaviour directed at Mixed stags over the course of the experiment is shown in Figure 7.7. During the pre-mixing 24 h profile no high intensity agonistic behaviours were recorded within the observation period (1500 to 2100 h). The frequency of low intensity agonistic interactions received over this period by Mixed stags was 0.08 ± 0.08 behaviours/h. There was no significant difference ($p = 0.457$) between mixing events (mix 1 to mix 6) in the frequency of high and low intensity aggression received by Mixed stags during the first 6 hours (15:00 to 21:00 h) of each event. The average frequency of low and high intensity aggression directed at Mixed stags during mixing was 0.87 ± 0.13 and 1.12 ± 0.12 behaviours/h/animal, respectively. The frequencies of both low and high intensity aggression were significantly higher ($p < 0.001$) than pre-mixing levels.

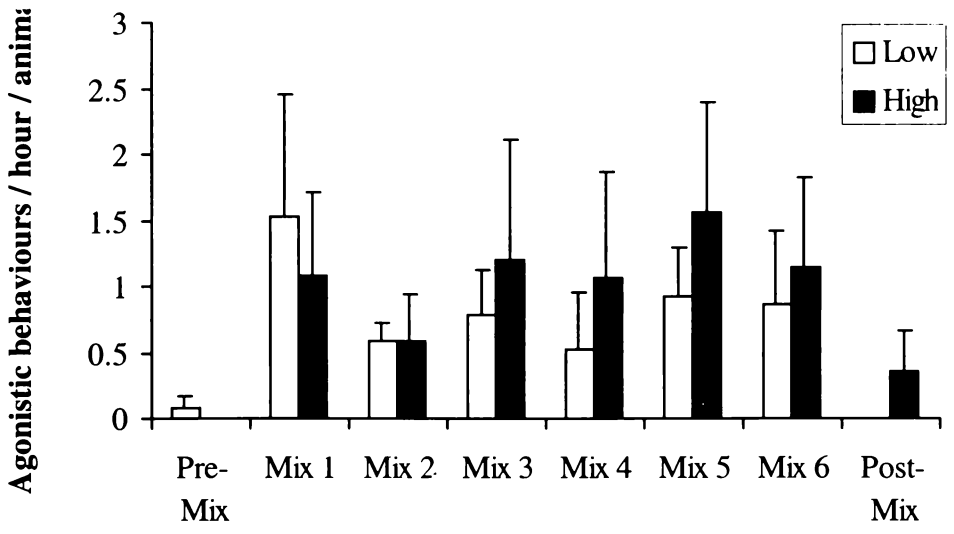


Figure 7.7 The frequency of low and high intensity agonistic behaviours received by Mixed stags during observations made on the first day (1500 to 2100 h) of the pre-mixing 24 h profile, mix events 1 to 6 and the post-mixing 24 h profile.

The rate of agonistic activity was highest ($p < 0.01$) in the first hour of mixing averaging over all mixing events 4.39 ± 0.63 and 2.92 ± 0.56 agonistic behaviours/h/animal for high and low intensity agonistic interactions, respectively (Figure 7.8). These levels of aggression declined to a minimum of 0.11 ± 0.08 and 0.19 ± 0.12 agonistic behaviours/h/animal, for high and low intensity interactions, respectively, by 2.5 h after introduction into the group. Mixed stags were still subjected to both high (0.34 ± 0.15 behaviours/h/animal) and low (0.18 ± 0.07 behaviours/h/animal) intensity aggression on the second day after mixing (18:00 to 21:00 h) of each mixing event.

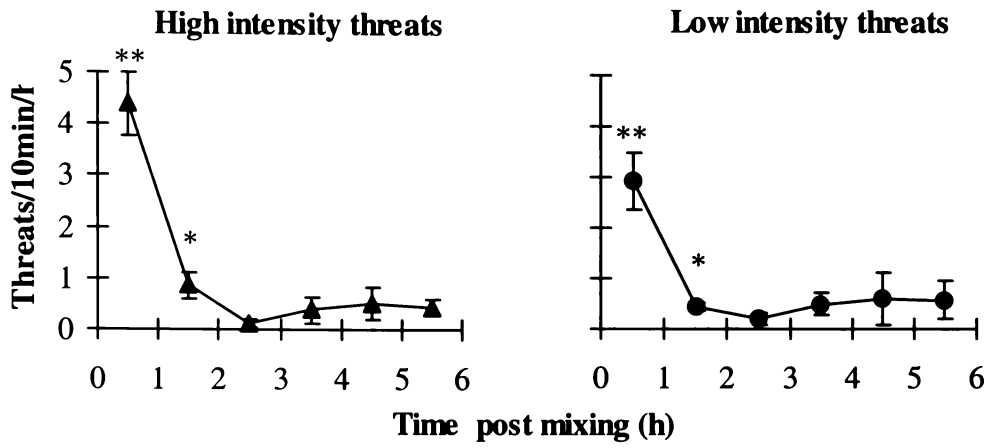


Figure 7.8 The mean number of high and low intensity threats received by Mixed stags during the 10 min of focal observations every hour, following mixing. The graphs show values averaged for each hour after mixing averaged over the 6 mixing events. Asterisks represent significant differences between subsequent hours (* $p < 0.05$, ** $p < 0.01$).

7.4.8.2 Maintenance activities

The effects of mixing on proportion of time spent performing general maintenance activities (lying, grazing, standing and walking) in Mixed stags and Base herd controls are shown in Figure 7.9 and described in the following section.

7.4.8.2.1 Lying

There was no significant difference between Mixed and Base herd controls in the proportion of time spent lying during the first 6 h (1500 to 2100 h) after mixing for any of the mixing events (Figure 7.9). The proportion of time spent lying remained consistent over the 6 mixing events for both Mixed and Base herd controls, overall mean proportion of time 0.46 ± 0.04 and 0.44 ± 0.03, respectively. The proportions of time spent lying by Mixed stags during the pre-mixing baseline observations. These pre-mixing levels of lying (0.42 ± 0.09) were similar ($p = 0.609$) to levels seen in Mixed stags during mixing.

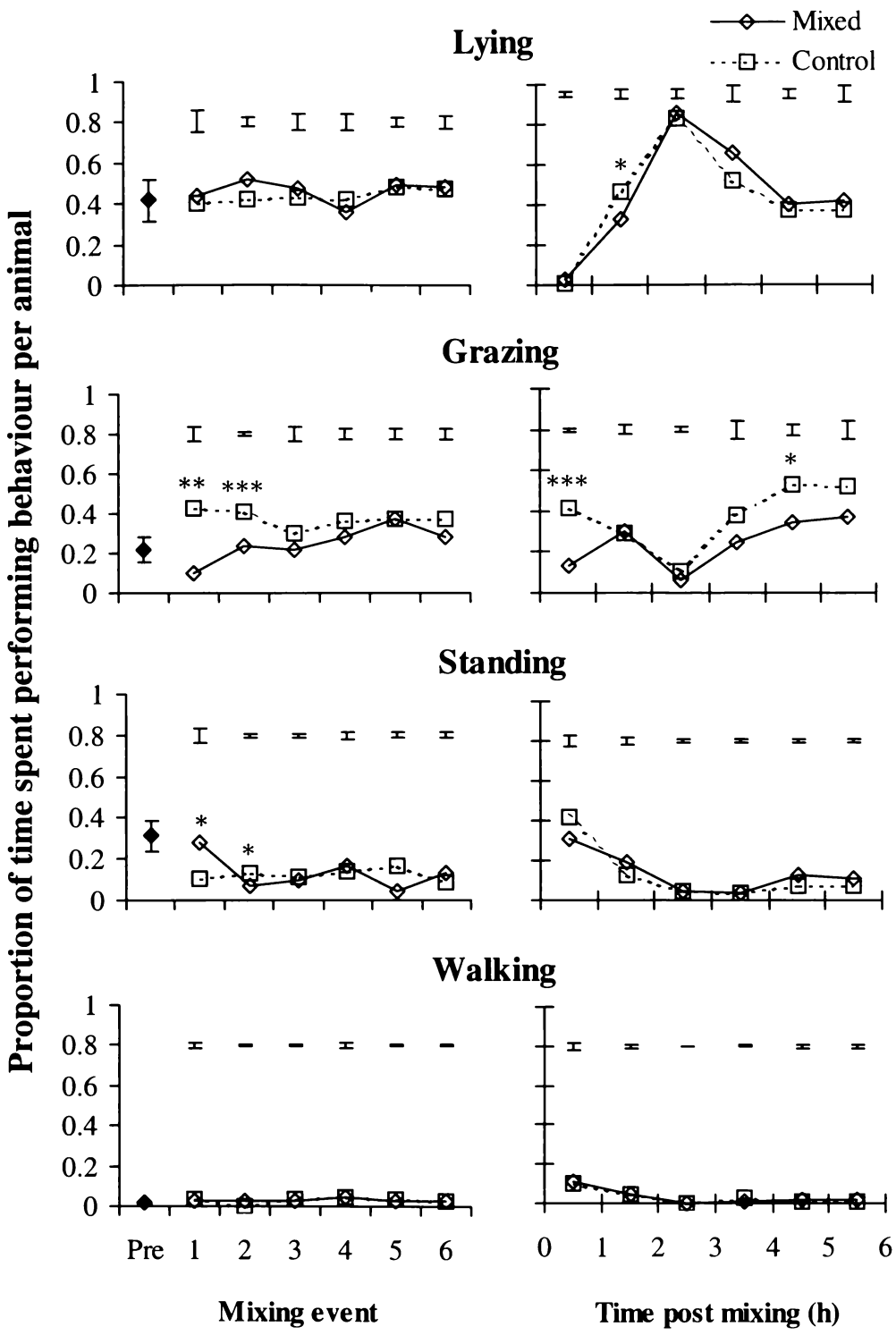


Figure 7.9 The proportion of time spent lying, grazing, standing and walking by Mixed (Mixed) and Base herd control (Control) stags. Graphs show values averaged over the whole of the observation period (1500 to 2100, Day 1) for the Pre-Mix (Mixed stags only) and each mixing event (Column A), and for each hour after mixing averaged over the 6 mixing events (Column B). Asterisks represent significant differences between treatments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Compared with Base herd controls, Mixed stags spent significantly ($p < 0.05$) more time lying (0.51 ± 0.04) on the second day after each mixing than Base herd controls (0.35 ± 0.05). The pattern of lying behaviour within mixing events is also shown in Figure 7.9. The pattern of change in lying behaviour over the 6 h period following mixing averaged over the 6 mixing events, was similar for Mixed and Base herd controls, the exception was that at 1.5 h post mixing Base herd controls spent significantly ($p = 0.020$) more time lying than Mixed stags. The proportion of time spent lying increased from very low levels 0.5 h post mixing to peak levels for both Mixed and Base herd controls 2.5 h after returning to pasture.

7.4.8.2.2 Grazing

Mixed stags spent significantly less time grazing during the first 6 h (1500 to 2100 h) for the first ($p = 0.006$) and second ($p = 0.002$) mixing events, than Base herd controls. There was no significant differences in grazing times between treatments for subsequent mixes (Figure 7.9). The average time spent grazing over the 6 mixing events for Mixed stags (0.25 ± 0.03) was also significantly less ($p = 0.015$) than that obtained for Base herd controls (0.37 ± 0.02). The levels of grazing seen in Mixed stags during mixing were not significantly different from their levels during the pre-mixing baseline observations (0.22 ± 0.03). The amount of time spent grazing varied significantly with mixing event with Mixed stag values increasing significantly ($p < 0.05$) from mix 1 to 2 and 3 to 4 while the values for Base herd controls increased significantly ($p < 0.05$) from mix 3 to 4. Mixed stags continued to spend significantly ($p < 0.01$) less time grazing (0.33 ± 0.04) on the second day of mixing than Base herd controls (0.50 ± 0.04). The average pattern of grazing behaviour during the first 6 h of each mixing event is shown in Figure 7.9. Significantly less ($p < 0.001$) grazing was performed by Mixed stags in the first hour post mixing than Base herd controls. The pattern of change in grazing behaviour was, however, similar after the first hour, with low levels of grazing seen in Mixed and Base herd controls 2.5 h after mixing. The amount of time spent grazing increased thereafter, though the extent of this increase was significantly ($p < 0.05$) greater in Base herd controls at 4.5 h post mixing.

7.4.8.2.3 Standing

Mixed stags spent significantly more of their total time standing than Base herd controls for mix 1 ($p = 0.050$) and significantly less time for mix 2 ($p < 0.05$). There was no significant differences in standing times for later mixes (Figure 7.9) or for the average value obtained from all mixing events (Mixed stags 0.13 ± 0.01, Base herd controls 0.12 ± 0.02, $p = 0.191$). Similarly time spent standing on the second day of mixing was similar for both Mixed (0.04 ± 0.01) and Base herd controls (0.04 ± 0.01). The average pre-mixing level of standing (0.31 ± 0.08) for Mixed stags was significantly greater than the average level during mixing ($p = 0.050$).

The pattern of standing behaviour within mixing events is shown in Figure 7.9. Mixed and Base herd controls exhibited similar values and a similar pattern of change ($p < 0.001$) in standing behaviour over the 6 h period following mixing. The levels of standing declined over time in both Mixed and Base herd controls to reach a nadir after 2.5 h.

7.4.8.2.4 Walking

The average time spent walking remained consistent across mixing events ($p = 0.536$) for both Mixed and Base herd controls (Figure 7.9). When averaged across mixing events the time spent walking on the first day of mixing by Mixed stags (0.03 ± 0.01) was similar ($p = 0.553$) to that spent walking by Base herd controls (0.03 ± 0.01). This was also the case for the second day of mixing (Mixed stags 0.003 ± 0.002, Base herd controls 0.017 ± 0.004, $p = 0.060$). Proportions of time spent walking by Mixed stags during the pre-mixing baseline observations (0.08 ± 0.04) were also similar ($p = 0.238$) to mixing values.

The pattern of walking behaviour within mixing events is shown in Figure 7.9. Mixed stags and Base herd controls exhibited both similar values and a similar pattern of change ($p < 0.001$) in walking behaviour over the 6 h period following mixing. Levels of walking declined over time in both Mixed and Base herd controls to a minimum 2.5 h post mixing.

7.4.8.3 Fence pacing

The average proportion of time spent fence pacing on the first day of mixing was similar ($p = 0.273$) over the 6 mixing events for Mixed and Base herd control stags with generally numerically higher values in Mixed stags, though these only reached significance ($p = 0.05$) for mix 5 (Figure 7.10). When averaged over all mixing events Mixed stags spent significantly more time ($p = 0.05$) fence pacing (0.11 ± 0.02) than Base herd controls (0.04 ± 0.02) or Mixed stags prior to mixing (0.03 ± 0.02, $p < 0.01$).

The average pattern of fence pacing behaviour within mixing events is shown in Figure 7.10. Levels of fence pacing were significantly higher ($p < 0.01$) in Mixed stags during the first hour of mixing and declined over time to reach a minimum values 2.5 h after introduction into the group.

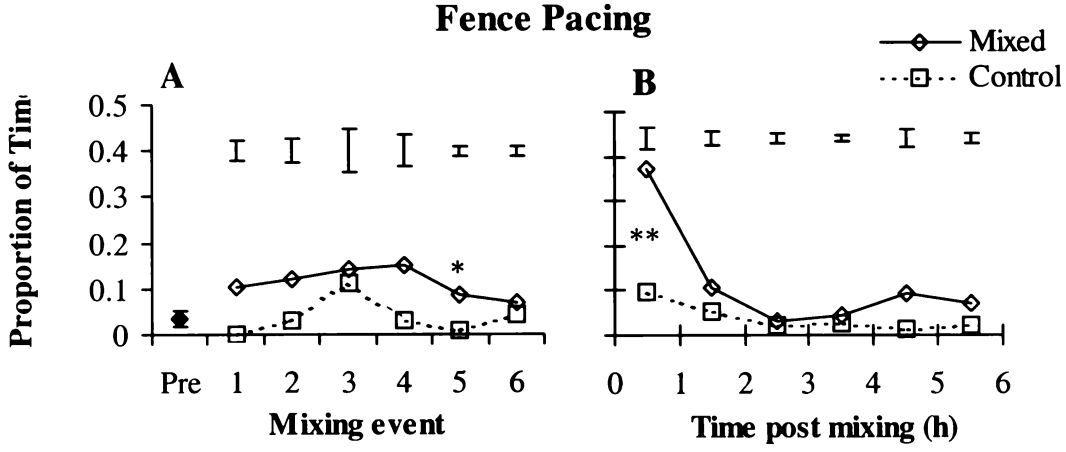


Figure 7.10 The proportion of time spent fence pacing by Mixed and Base herd control (Control) stags during mixing. Graphs show values averaged over the whole of the observation period (1500 to 2100) during the pre-mix (Mixed stags only) and each mixing event (A), and for each hour after mixing averaged over the 6 mixing events (B). Asterisks (* $p < 0.05$, ** $p < 0.01$) represent significant differences between treatments.

Levels of fence pacing in Base herd controls remained at a consistently low level throughout the 6 h period. Fence pacing was also observed on the second day of each mixing event with significantly more ($p < 0.05$) fence pacing seen in Mixed stags (0.04 ± 0.01) than Base herd controls (0.006 ± 0.003).

7.4.8.4 Nearest neighbour

The distance between Mixed stags and their nearest neighbour (NN) was significantly greater ($p < 0.001$) during the first 6 h of each mixing event (overall mean, 3.12 ± 0.09 NN units) compared with distances recorded during the pre-mixing period (2.30 ± 0.12 NN units). During the first 6 h of each mixing event, NN distances were significantly greater ($p < 0.001$) for Mixed stags than Base herd controls (overall mean, 1.76 ± 0.03 NN units). NN distances were also significantly greater ($p < 0.001$) on the second day and during the post-mixing 24 h profile (day 5 of Mix 6) for Mixed stags (3.07 ± 0.09 and 3.25 ± 0.12 NN units, respectively) than Base herd controls (2.07 ± 0.05 and 1.76 ± 0.15 NN units, respectively). NN distances remained consistent across mixing events for both Mixed stags and Base herd controls (Figure 7.11).

The pattern of change in NN distances within mixing events is shown in Figure 7.11. NN distances were consistently greater for Mixed stags during the first 6 h of mixing. NN distances increased significantly ($p < 0.01$) after the first hour for Mixed stags and remained consistent after that, whereas, NN distances of Base herd controls declined significantly ($p < 0.05$) at 2.5 h post mixing corresponding to the peak in lying, but increased again ($p < 0.01$) by 3.5 hours post mixing.

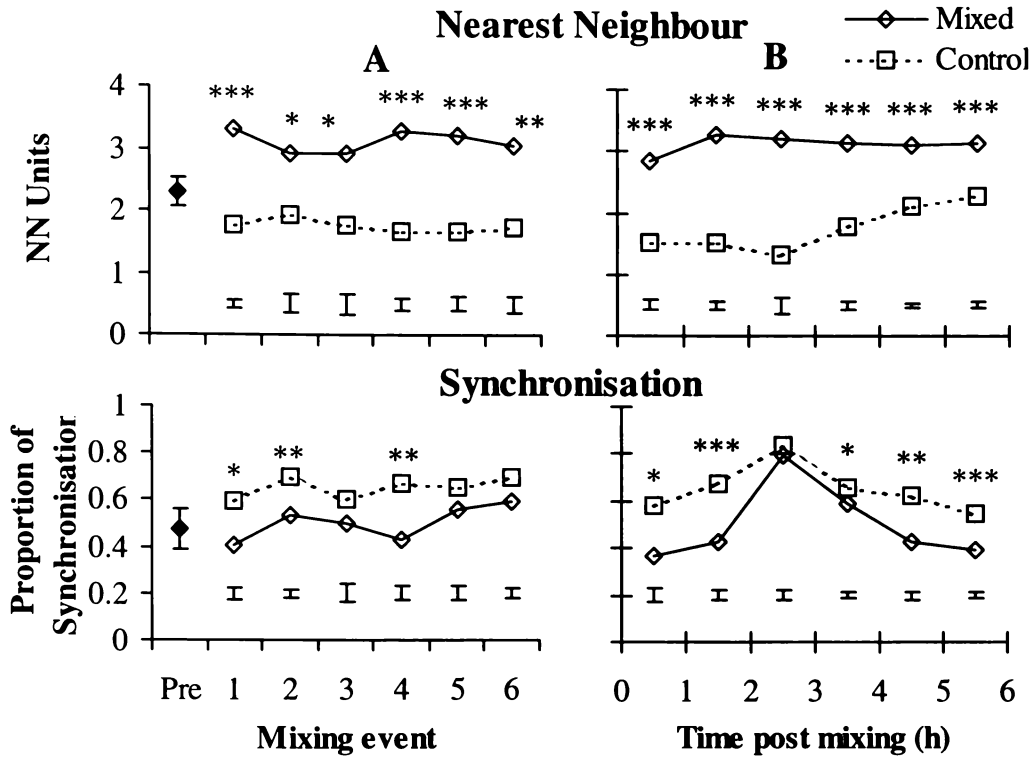


Figure 7.11 The degree of spatial and temporal cohesion exhibited by Mixed and Base herd controls with the rest of the group as measured by nearest neighbour distance (non linear scale, NN units) and the temporal synchronisation of behaviour (proportion of remainder of group performing same behaviour as the Mixed stags or Base herd controls). Graphs show values averaged over the whole of the observation period (1500 to 2100) for the pre-mix (Mixed stags only) and each mixing event (column A), and for each hour after mixing averaged over the 6 mixing events (column B). Asterisks (* $p < 0.05$, ** $p < 0.01$) represent significant differences between Mixed and Base herd control animals.

7.4.8.5 Behavioural synchronisation

Temporal synchronisation of behaviour calculated for each mixing event was consistently higher in Base herd controls than Mixed stags with differences reaching significance ($p < 0.05$) for mixes 1, 2 and 4 (Figure 7.11). Levels of synchronisation also varied significantly ($p < 0.01$) over the 6 mixing events with the lowest levels recorded during mix 1 for both Mixed and Base herd control stags. When averaged over all mixing events the proportion of behavioural synchronisation of Mixed stags to the rest of the group was (0.51 ± 0.02) during the first 6 h period of mixing. This was significantly less ($p < 0.001$) than the levels seen in the Base herd controls (0.66 ± 0.03). A similar response was seen

on the second day of mixing with levels of synchronisation in Mixed stags (0.49 ± 0.03) significantly less ($p = 0.001$) than Base herd controls (0.64 ± 0.02). However, the levels of synchronisation seen in Mixed stags during mixing were not significantly different from their levels during the pre-mixing baseline observation period (0.47 ± 0.02).

The pattern of change in temporal synchronisation of behaviour within mixing events is shown in Figure 7.11. Synchronisation of behaviour was significantly less ($p < 0.001$) in Mixed stags throughout the 6 h observation period apart from the values obtained 2.5 h after mixing. The level of behavioural synchronisation seen in both Mixed and Base herd controls remained similar for the first 2 h but then increased significantly ($p < 0.050$) between the second and third hours. This increase corresponded with the increase in lying behaviour seen in Mixed and Base herd control stags. Values then declined to levels which were similar to those at the start of mixing.

7.5 DISCUSSION

This study has shown that repeated mixing of red deer stags into unfamiliar groups results in both acute and long-term changes in behaviour, HPA axis activity and function and weight gain. The acute response to mixing is characterised in the first few hours by a strong activation of the HPA axis and behavioural responses indicative of disturbance (e.g. fence pacing). This is followed by an ongoing lower level of disruption in some activities and an apparent normalisation of some parameters of HPA axis activity. However, other measures of HPA axis basal activity, plasma cortisol binding capacity and responsiveness to ACTH and CRH challenge following repeated mixing are consistent with significant changes in the activity and functioning of the HPA axis indicative of chronic stress.

7.5.1 The acute response to mixing

Mixing of unfamiliar animals into established groups of stags resulted in an increase in aggression directed at the newcomers and a general increase in

responses (e.g. fence pacing) which suggested the newly introduced deer were attempting to escape the group. Similar changes have previously been reported for individual red deer mixed with unfamiliar animals in pens (Kay *et al.* 1981, Pollard *et al.* 1993, Hanlon *et al.* 1995). Perturbations to normal maintenance activities were also observed during the initial response to mixing (e.g. less lying and grazing and higher frequencies of standing and walking). These changes in maintenance activities are consistent with those reported for red deer stags following the stress of surgical removal of velvet antler (Matthews *et al.* 1990) and yarding and handling procedures (Carragher *et al.* 1997). High levels of behavioural disturbance have been reported to occur following periods of stressful handling in red deer (Matthews *et al.* 1990, Pollard *et al.* 1993, Diverio *et al.* 1996a, Carragher *et al.* 1997). Therefore the initial behavioural changes seen in Mixed stags upon return to the paddock may reflect the stress associated with the mixing process (yarding and drafting) in addition to any social stress component of the mixing procedure. However, the greater level of behavioural disturbance seen in Mixed stags compared with Base herd controls would suggest that the stress associated with introduction into a unfamiliar herd is a major factor.

A strong activation of the HPA axis was also apparent during the initial phase of mixing. Plasma cortisol concentrations increased rapidly during the yarding and drafting process, with maximum concentrations obtained around the time animals were returned to pasture (approx. 1 h after the start of the mixing procedure). The timing of this response and the rapid decline once animals entered their paddocks could suggest that the stress associated with the yarding and drafting of these animals was the main factor contributing to the initial activation of the HPA axis. However, the maximum cortisol concentrations obtained during the initial phase of mixing (approximately 50 ng/ml) were consistently higher than those previously reported for red deer stags subjected to yarding and drafting in our facility at a similar time of year (23-30 ng/ml, Carragher *et al.* 1997, Ingram *et al.* 1999, Chapter 5). Therefore it appears that mixing into novel groups while in the yards results in a greater HPA axis response than would be expected from just yarding and drafting alone. Indeed, the maximum response of the HPA axis in the Mixed stags to the mixing procedure was similar to that seen during exposure to

intensive handling procedures such as physical restraint in a drop floor crush (46-56 ng/ml, Ingram *et al.* 1994, 1997, Carragher *et al.* 1997, Chapters 3 & 4). In red deer stags, greater plasma cortisol responses are associated with more intensive handling procedures and may reflect the animal's perception of the severity of the stressor imposed (Carragher *et al.* 1997). Thus, mixing at least in the initial phase would appear to represent a high intensity stressor for the Mixed stags.

The maximum cortisol response to mixing did not differ between the first and final mixing events, though cortisol concentrations immediately prior to mixing and during recovery at pasture tended to be lower during the final mixing. The response to repeated stressors depends upon a variety of factors such as intensity, duration, number of repetitions, and frequency of the application (Natelson *et al.* 1988). With repeated exposure to less intensive stressors, the stress response may decline (habituation), whereas with more intensive or painful stressors the stress response generally increases upon repeated exposure (sensitisation) (Konarsky *et al.* 1990, Pitman *et al.* 1990). The lack of habituation of both the behavioural and HPA axis responses to mixing provides further evidence that the initial phase of each mixing was perceived as a relatively intense stressor by the Mixed stags.

Average cortisol concentrations declined rapidly once animals were returned to their respective paddocks, reaching premixing baseline values within an hour. This decline was similar to changes seen in many of the behavioural measures including levels of aggression and fence pacing. Disruption of social hierarchies has been reported to increase plasma glucocorticoids concentrations (Alexander & Irvine 1998, Dalin *et al.*, 1993, Mench *et al.* 1990, Tsuma *et al.* 1996) which remain elevated while the social hierarchy is unstable (Sapolsky 1990, Shively *et al.* 1997). Once a stable social hierarchy is established basal concentrations of glucocorticoids in subordinate animals can remain chronically elevated (Blanchard *et al.*1993, Sapolsky 1982, Shively *et al.* 1997, Schuhr 1987) or decline to levels similar to (Smith & French 1997, Mays *et al.* 1991) or lower than those of dominant conspecifics (Creel *et al.* 1996, Pollard & Littlejohn 1998, Saltzman *et al.* 1998). During the first mixing event the activity of the HPA axis following the return of the Mixed stags to pasture exhibited considerable individual variability. This variability can be attributed to secondary peaks in

cortisol seen in the cortisol profiles of a number of individuals at different times following their return to pasture. These secondary peaks in cortisol concentrations appear in some instances to be correlated with periods of agonistic behaviour and thus could be indicative of social stress. This variability was not so evident during the final mixing event and may represent some adaptive process, either behavioural or physiological, by which Mixed stags reduced the affects of social stress associated with mixing. For example, in later mixing events Mixed stags were observed lying in hollows or patches of longer grass within the paddock, possibly in an attempt to reduce levels of aggression by making themselves less conspicuous to other members of the group.

Within each mixing event some of the behavioural measures of stress declined following the initial response to mixing. Levels of aggression and fence pacing declined while the proportion of time spent lying and grazing increased. The decline in aggression occurred within the first hour of mixing, but levels remained above pre-mixing values throughout the 2 days of each mixing event. Other behavioural changes evident in this secondary phase of the response to mixing may have also (see above) played a role in reducing aggression. Mixed stags may have reduced social contact and hence opportunities for agonistic interactions by distancing themselves from other members of the herd, as evidenced by a significant increase in NN distance by the second hour of mixing. This approach to avoiding aggression may have been limited by the confines of the experimental paddocks as levels of aggression remained above pre-mixing levels throughout the second day of each mixing event.

Mixed stags generally spent less time grazing particularly following the first and second mixing. This difference was also evident on the second day of each mixing event. The decline in grazing may account for some of the dramatic loss in live weight seen in the Mixed stags by the end of the trial. Repeated mixing with unfamiliar conspecifics causes substantial depression of growth in pigs (Stookey & Gonyou 1994) yet a previous report on mixing of yearling red deer hinds found no effect on live weight (Hanlon *et al.* 1995). Stress can induce suppression of appetite and feeding behaviour via increased activity of CRH containing neurons in the paraventricular nucleus (PVN) of the hypothalamus

(Morley *et al.* 1986). Interestingly human patients with anorexia nervosa have a reduced response to CRH challenge (Gold *et al.* 1984) as did chronically stressed stags in the present study suggesting a role of CRH in mediating the effects seen in the present study. Base herd stags also displayed a depression in growth compared with unhandled controls. This reduction in growth may have resulted from stress associated with the mixing procedure (e.g. repeated handling, introduction of an unfamiliar stag into the group).

Male red deer are essentially gregarious in nature forming bachelor herds for most of the year (Clutton-Brock *et al.* 1982). Particularly in open habitats, formation of groups by deer is believed to be an adaptation against predation (Clutton-Brock *et al.* 1982). The temporal synchronisation of behaviours between individuals within a group is an important factor in maintaining spatial cohesiveness and group structure (Rook & Penning 1991). Behavioural activities in undisturbed red deer show clear signs of being socially facilitated and this may influence cohesion within groups (Clutton-Brock *et al.* 1982). In addition, non-conformance to group activity patterns is considered to be a sensitive indicator of an individual's well being (Friend 1991). The behavioural activity of Mixed stags lacked the high degree of temporal synchronisation with the rest of the group evident in the behaviour of Base herd controls over the 2 days of each mixing event (apart from during the peak in lying behaviour at 2-3 h post mixing). Both the lower spatial cohesiveness (greater NN distance) and temporal synchronisation of activities would suggest that Mixed stags did not assimilate easily into the unfamiliar social group over the two day mixing period. This form of social isolation remained consistent over time and may have accounted for much of the chronic stress associated with repeated mixing, particularly as deer in farm situations exhibit a strong motivation to be part of a group.

7.5.2 The long-term response to mixing

7.5.2.1 Basal activity of the HPA axis

Repeated mixing of animals produced significant changes in the activity and functioning of the HPA axis, including changes in basal activity, plasma cortisol

binding capacity and responsiveness to ACTH and CRH challenge. The basal activity of the HPA axis prior to mixing was similar to that previously reported for stags monitored at a similar time of year using the DracPac technique (November, Ingram *et al.* 1999, Chapters 5). Mean 24 h cortisol concentrations and cortisol pulse parameters were similar to those previously reported in Chapter 5 (e.g. 24 h mean concentration, 12.9 ± 1.5 and 12.5 ± 1.0 ng/ml; cortisol pulse height, 17.8 ± 1.6 ng/ml and 17.1 ± 1.3 ng/ml; amplitude, 9.19 ± 0.9 ng/ml and 9.7 ± 0.8 ng/ml; nadir 8.4 ± 1.2 and 7.5 ± 0.6 ng/ml, respectively). Cortisol pulse frequencies obtained in the present study were also similar to those reported for Eld's deer (0.6 peaks/h, Monfort *et al.*, 1993), and other ruminant species including cattle (0.5 peaks/h, Ladewig & Smidt 1989). This would suggest that basal activity prior to mixing was typical of normal activity in stags for this time of year.

Following application of the repeated mixing stressor mean 24 h cortisol concentrations declined as did cortisol pulse parameters such as pulse height and nadir. Unfortunately basal activity could only be compared between 3 Mixed stags prior to and following mixing due to equipment failure. However, baseline measures obtained during both the final mixing and the post-mixing ACTH challenge also indicated a decline in basal cortisol secretion. Declining basal cortisol concentrations have also been reported for young red deer subjected to repeated mixing stress (Hanlon *et al.* 1995) and during habituation to indoor housing (Goddard *et al.* 1994) and therefore may be a common feature of the response to chronic stress in red deer.

7.5.2.2 Circadian rhythm

A circadian rhythm in 24 h profiles of plasma cortisol was apparent in the present study both prior to (5 out of 6) and following (2 out of 3) repeated mixing. The amplitude and acrophase were also similar before and after repeated mixing, but the mesor (around which the rhythm fluctuates) was significantly higher before mixing. Circadian rhythms in plasma cortisol concentrations have been found previously in red deer using the DracPac remote sampling technique (Ingram *et al.* 1999, Chapters 5 & 6) but have not been identified in other deer species using traditional blood sampling techniques (e.g. white-tailed deer, Bubenik *et al.* 1983;

rusa deer, van Mourik & Stelmasiak 1984; and Eld's deer, Monfort *et al.* 1993). This could suggest that circadian rhythms in deer are readily attenuated by handling stress. In other species chronic or repeated stress has also been shown to blunt circadian rhythmicity of glucocorticoids by increasing concentrations during the nadir (e.g. rats, Akana *et al.* 1992; horses, Irvine & Alexander 1994). It is therefore surprising, even with respect to the low overall numbers of animals, that cortisol profiles in two of the three Mixed stags exhibited a circadian rhythm following the repeated mixing stressor. However, the post mixing 24 h profiles where obtained on the fourth and fifth day of the final mixing event. By this time base herd members may have tolerated the presence of Mixed stags to the extent that social stress was no longer a significant source of disruption to the circadian rhythm in HPA axis activity. This hypothesis is supported by the observed decline in agonistic behaviour from the first day to the fifth day of the final mixing event (Figure 7.7). However, complete assimilation into the group is unlikely at this stage as NN distances for Mixed stags were still elevated.

7.5.2.3 Free cortisol

The lower basal cortisol concentrations seen in Mixed stags following the repeated mixing stress was accompanied by a decrease in plasma cortisol binding capacity. The net effect of this decline in plasma cortisol binding capacity was to keep free cortisol concentrations within the normal range despite the marked decline in total cortisol. Free cortisol concentrations were similar prior to and following mixing in samples containing low and high total cortisol concentrations. Interestingly, free cortisol concentrations in low and high cortisol samples were also similar to those measured in stags during the seasonal nadir in total cortisol concentrations during the breeding season (low 0.41 ± 0.21 ng/ml, high 7.62 ± 1.50 ng/ml, Chapter 6).

The cortisol binding capacity of plasma is largely determined by the concentrations of corticosteroid-binding globulin (CBG) which binds cortisol with much higher affinity and specificity than albumin and is the major determinant of cortisol bio-availability (Hammond 1997). In stags under resting conditions the percentage of CBG bound glucocorticoid is 72.5 ± 2.3 % with 19.4 ± 1.8 % bound

to albumin (J. R. Ingram unpublished data) which fall within the range reported for other domestic species (67 to 87 %, Gayrard *et al.* 1996). Both acute (Marti *et al.* 1993, 1995, Fleshner *et al.* 1995) and chronic stress (Kattesh *et al.* 1980, Blanchard *et al.*1993, Spencer *et al.* 1996, Alexander & Irvine 1998) can lower plasma cortisol binding capacity, though given the long circulatory half life of CBG (e.g. 14.5 h in rats, Smith & Hammond 1992) such changes are only apparent 24-48 h after stress (Fleshner *et al.* 1995, Dalin *et al.* 1993). In contrast to the present findings, declines in plasma cortisol binding capacity during social stress in rats (Blanchard *et al.* 1993) and horses (Alexander & Irvine 1998) and during fasting in cattle (Ward *et al.* 1992) are not accompanied by declines in total cortisol concentrations. Thus in these species the stress-induced fall in binding may function to increase free glucocorticoid concentrations thereby increasing their effect on target tissues (Fleshner *et al.* 1995). Whereas in the present study free cortisol concentrations were maintained at pre stress levels as a result of the observed decline in plasma cortisol binding capacity. It may be that this apparent difference between species is attributable to the time of measurement relative to the duration of the stress response or is a result of the different types, duration and intensities of chronic stress used in the various experiments. Jensen *et al.* (1996a) has proposed a process of adaptive "normalisation/suppression" of activity of the HPA axis during chronic stress. This involved an acute period of increased basal HPA axis activity is followed by an adaptive biochemical down-regulation at some level in the HPA axis in order to protect the organism from detrimental effects on growth, reproduction and immune status due to sustained high levels of glucocorticoids. In the present study plasma cortisol binding capacity was assessed in samples collected from stags 6 days after introduction into their final mixing group. The relatively low levels of aggression recorded during observations 2 days prior to this (24 h post mixing) would suggest that by the time they were sampled their presence in the group was no longer a significant source of social stress. It is likely that once stress induced stimulatory inputs to the HPA axis have ceased normal glucocorticoid inhibitory feedback takes over reducing total cortisol concentrations until free cortisol has returned to its set point (Orth *et al.* 1992). The cortisol binding capacity of plasma may take longer to return to normal levels, thus a period of low plasma cortisol binding capacity, low total

cortisol concentrations but normalised free cortisol concentrations may be characteristic of a recovery phase following chronic stress and not a response to it *per se*.

The mechanism by which stress reduces the plasma binding capacity for cortisol has yet to be determined in deer. However, stress may act by reducing the synthesis of CBG in the liver, increasing its clearance from the circulation or reducing the binding affinity of CBG for cortisol. Glucocorticoid levels may play a role as CBG concentrations increase following adrenalectomy and decrease following chronic high doses of exogenous glucocorticoids (Feldman *et al.* 1979, Smith & Hammond 1992). In addition, CBG synthesis is regulated by immune activation associated with the acute phase response (Savu *et al.* 1980). The acute phase response refers collectively to the physiological changes that are initiated immediately following pathogen infection or tissue trauma which include a shift in liver metabolism from the synthesis of carrier proteins (e.g. CBG) to production of acute phase proteins such as haptoglobin (Baumann & Gauldie 1994, Deak *et al.* 1997). An acute phase response can also be initiated by stress and is thought to represent an anticipatory defensive immune response which may restrict infection, inflammation and injury produced by the threat (Deak *et al.* 1997).

7.5.2.4 HPA function

Changes in HPA axis function were also apparent following mixing with reductions in peak cortisol concentrations and in the area under the curve in response to the ACTH and CRH challenges. Studies on other domestic species have shown that conditions of chronic stress can increase (Friend *et al.* 1977, 1979, 1985, Janssens *et al.* 1995, Sakellaris & Vernikos-Danellis 1975) or decrease (Dantzer *et al.* 1983, Ladewig & Smidt 1989, Rees *et al.* 1985) the adrenal responsiveness to ACTH. In red deer, the adrenal responsiveness to ACTH has been reported to increase relative to baseline cortisol concentrations during chronic social stress (Hanlon etal.1995) (actual peak cortisol concentrations remained the same as controls) or decrease during indoor housing (Goddard *et al.* 1994, 1996, Pollard & Littlejohn 1998). Adrenal responsiveness to ACTH also exhibits marked seasonal variation in red deer stags with lower

peak responses reported during the rut (Suttie *et al.* 1995, Cassidy 1996, Ingram *et al.* 1999, Chapter 5 & 6).

A number of possible mechanisms could account for the observed decline in adrenal responsiveness to exogenous ACTH in the present study. The observed reduction in plasma cortisol binding may increase free cortisol inhibitory feedback upon the adrenal cortex. However, direct inhibition of adrenal steroidogenesis only occurs at extremely high levels of cortisol (Labhart 1986). Prolonged inhibition of endogenous ACTH release, whether from reduced pituitary responsiveness to CRH or a reduced activity of the higher centres of the HPA axis, may lead to the loss of trophic support to the adrenal resulting in atrophy of the adrenal cortex and reduced responsiveness to ACTH (Labhart 1986). Alternatively, stress induced hypersecretion of ACTH from the pituitary may result in ACTH induced desensitisation of adrenal cells, as has been reported in rats (Rani *et al.*1983). Increased clearance rates of cortisol may have also have accounted for the decline in response to ACTH as circulating cortisol concentrations are influenced by the rate of hormone metabolism as well as synthesis. However, clearance rates of cortisol following ACTH challenge were similar prior to and following chronic social stress. Thus reduced cortisol concentrations, both baseline and ACTH stimulated, following chronic stress are likely to reflect a reduced rate of synthesis in the adrenal cortex rather than an increased rate of clearance from the circulation.

The significance of the reduction in adrenal function is difficult to interpret, as the biologically active component of the response, the concentration of free cortisol, did not differ before or after the repeated mixing. However, as a measure of chronic stress, the remotely administered ACTH challenge test was useful in quantifying significant changes in adrenal function following chronic stress

Although endogenous ACTH secretion was not measured following the CRH challenge, a reduced pituitary responsiveness to CRH following repeated mixing stress is implied, as cortisol responses were considerably lower. The decline in peak cortisol responses to CRH post-mixing (53.9 ± 7.3 % of the pre-mixing value) tended to be greater ($p = 0.067$) than the decline in response associated

with the ACTH challenge (77.3 ± 3.8 % of the pre-mixing value). Thus repeated mixing stress is likely to have produced changes in HPA axis function at both the level the pituitary and adrenal glands. Chronic social stress in horses has been shown to reduce pituitary responsiveness (ACTH secretion) to exogenous CRH challenge and to increase pituitary venous blood concentrations of CRH in animals at rest (Alexander *et al.* 1996). Endogenous ACTH secretion was also reduced in these horses, and in pigs subjected to 6 days of intermittent stress (Jensen *et al.* 1996b). Reduced pituitary responsiveness to CRH is also characteristic of a number of human psychiatric disorders such as depression, panic disorder, anorexia nervosa and post-traumatic stress disorder (Smith *et al.* 1989). In the present study pituitary responsiveness to CRH may have also been influenced by the suppressive effects of increased glucocorticoid concentrations associated with the preceding ACTH challenge. However, as glucocorticoid feedback and free cortisol concentrations in response to ACTH remained unchanged following mixing it is unlikely that this effect contributed to the observed difference in response.

The CRH challenge test was effective at quantifying changes in pituitary function in red deer even though maximal stimulation of the HPA axis was not achieved with the dose of CRH used. It has been demonstrated in rats following chronic stress that ACTH responses are attenuated to low but not high doses of CRH (Odio & Brodish 1990). Therefore, the low dose of CRH used in the present study may serve as a more sensitive measure of changing pituitary function during chronic stress in deer than higher doses.

An interesting feature of the response to CRH challenge in the present study was a biphasic response seen in the pre-mixing CRH challenge. A biphasic response to CRH challenge has also been reported in other species (Orth *et al.* 1983, Saphier *et al.* 1992), but was not a feature evident in a previous study using the same dose of CRH on stags (Chapter 6). The biphasic response observed in stags prior to application of the chronic stressor may be a function of the sub-maximal dose of CRH used. Though CRH has been reported to directly stimulate glucocorticoid secretion from the adrenal cortex of a number of species including humans (Fehm *et al.* 1988), rats (Mazzocchi *et al.* 1989) and cattle (Carroll *et al.* 1996, Jones &

Edwards 1990). Therefore the biphasic peak may reflect temporal differences in the stimulation of the adrenal and the pituitary by CRH. It is interesting to note that after chronic social stress the latter larger peak was completely abolished.

The mechanism by which chronic social stress reduced pituitary responsiveness to CRH has yet to be determined in red deer. However, both hyper and hypo-secretion of CRH secretion from parvocellular neurones of the paraventricular (PVN) nucleus into the hypophyseal portal system have been implicated in human psychiatric disorders such as depression, anorexia nervosa and posttraumatic stress disorder which exhibit reduced pituitary responsiveness to CRH (Chrousos 1995). Anterior pituitary responsiveness to CRH could therefore be influenced by either a reduction in trophic support to the pituitary during hypoactivity of the HPA axis or a down regulation of CRH receptors as seen in subordinate male tree shrews with hyperactivity of the HPA axis following chronic psychosocial stress (Fuchs & Flügge 1995). Hyperactivity of the HPA axis may also increase glucocorticoid inhibitory feedback upon the anterior pituitary particularly as plasma cortisol binding is reduced following chronic stress. Further research is required investigating endogenous CRH and ACTH secretion during chronic social stress in deer before the changes seen in pituitary function can be attributed to increased or decreased activity of higher levels of the HPA axis.

7.5.2.5 Dexamethasone

Differences in baseline total cortisol concentrations prior to dexamethasone challenge were evident between Mixed and Unmixed controls. This may reflect a process of habituation to handling by Mixed stags or a general decline in their ability to mount an HPA axis response to stress. The latter explanation would appear unlikely as Mixed stags were still able to mount a robust HPA axis response to the handling involved in the final mixing event. Apart from the reduced baseline cortisol concentration, there was little differences between Mixed stags and Unmixed control in response to dexamethasone. The slope describing the rate of decline in cortisol was lower in Mixed stags following dexamethasone but this may have reflected the lower cortisol levels Mixed stags had to start with. Since both Mixed and Unmixed controls had similar responses

to dexamethasone, there was no evidence of resistance to dexamethasone suppression. However, impaired glucocorticoid negative feedback is normally associated with glucocorticoid hypersecretion. Mixed stags subjected to repeated mixing stress exhibited hypo-secretion of glucocorticoids at the time of testing, a condition unlikely to result in impairment of glucocorticoid mediated negative feedback.

7.5.3 Conclusion

The duration of the HPA axis response to mixing would suggest that the stress imposed with repeated mixing was acute with a rapid return to basal levels corresponding with the decline in aggression. However the long term behavioural changes such as the reduction in grazing, increased nearest neighbour distances and reduced behavioural synchronisation with the group would suggest that some aspects of the stress imposed was more persistent in nature. In support of this chronic changes were also observed in the basal activity and functioning of the HPA axis when animals were monitored after the final mixing event.

Taken together these data support the model proposed by Jensen *et al.* (1996a) where chronic stress is characterised by an acute period of increased basal HPA axis activity is followed by an adaptive biochemical down-regulation at some level in the HPA axis in order to protect the organism from detrimental effects on growth, reproduction and immune status due to sustained high levels of glucocorticoids.

In summary, repeated mixing of individual red deer stags into unfamiliar herds resulted in dramatic weight loss and changes in both behaviour and HPA axis activity and function indicative of stress. Introduced animals were subjected to increased levels of aggression and exhibited reduced grazing and increased pacing of fence lines. They also attempted to distance themselves from the rest of the herd and were less synchronised in their behaviour with others in the group. The initial response to mixing involved activation of the HPA axis. However, long term changes in HPA axis activity and function were characterised by a lowering of basal cortisol concentrations (hypocortisolemia), an increase in the proportion

of unbound cortisol (although free cortisol concentrations did not change) and a reduction in the responsiveness of the adrenal to ACTH and the pituitary to CRH. Therefore, one must conclude that repeated mixing into unfamiliar herds is stressful for red deer stags and results in both acute and chronic changes in physiology and behaviour. Chronic social stress induced by repeated mixing into unfamiliar herds offers a potential model for assessing the impacts of chronic stress on aspects of deer physiology, immunology and behaviour.

CHAPTER 8 GENERAL DISCUSSION

There is an increasing awareness of animal welfare related issues in the farming of red deer and the need for improvements in animal husbandry procedures to better suit the requirements of these animals. As a possible consequence of their relatively recent domestication, farmed red deer still maintain much of the "flighty" nature characteristic of their wild conspecifics. This is evident in their behavioural and physiological reactivity to both acute and chronic stress, which has been linked to poor reproductive performance and increased susceptibility to infectious disease.

The reduction of stress is an important component of improved management, reducing animal welfare problems and increasing the efficiency of production systems. However, achieving a reduction in management stress requires reliable, quantifiable measures of stress relevant to the species in question. Changes in the activity and function of the hypothalamic-pituitary-adrenal (HPA) axis are routinely used to assess the response to stress in farm animals. However, little is known about the activity and functioning of the HPA axis and how these may change in response to stress in red deer. The overall aim of the research was therefore to investigate the activity and functioning of the HPA axis in red deer stags under basal and stress induced conditions.

8.1.1 The DracPac remote blood sampling technique

A number of studies have demonstrated that measurements of the HPA axis are readily confounded by the stress associated with handling and restraint of animals using traditional blood sampling methods (Chapter 3). An effect that is further

exacerbated in wild or semi-domesticated animals or in species with "flighty" natures such as red deer. Therefore the initial focus of the research was to develop remote blood sampling technology as a low stress alternative to traditional blood sampling techniques. The development of the DracPac remote blood sampling device allowed the collection of blood samples from undisturbed animals at pasture and the remote administration of bolus infusions of ACTH or CRH and monitoring of the subsequent adrenocortical response. This method has an advantage over traditional blood sampling in that the requirement for repeated handling is removed, though some degree of habituation to carrying the sampling equipment is required. The size of the DracPac system and the requirement to store the blood sample on the animal until retrieval does, however, impose limitations upon the size of animal that can be remotely sampled as well as restricting any measurement to compounds that are relatively stable over time in whole blood. However, remote blood sampling does lend itself to the monitoring of glucocorticoid concentrations, which are relatively stable in whole blood for several days (Reimers *et al.* 1983), and the sampling of large, "flighty", free ranging animals such as red deer. The low stress nature of the sample collection using this technique is highlighted by the fact that basal plasma cortisol concentrations were similar to those reported for undisturbed red deer stags shot in the field (Smith & Dobson 1990). By removing the confounding effects of handling, the interpretation of HPA axis responses to stress can be more readily quantified. The DracPac technique has since been applied successfully in the assessment of a wide range of stressors in both deer (velveting, Matthews *et al.* 1994; handling, Ingram *et al.* 1994, 1997, 1999, Carragher *et al.* 1997; transport, Waas *et al.* 1997, 1999) and cattle (surgical castration, Carragher *et al.* 1996). The effects of handling can also confound the interpretation of responses to tests of HPA axis function. The low stress nature of the sample collection permits the use of low dose ACTH and CRH challenges which have previously been shown to be more sensitive for detecting subtle changes in adrenal and pituitary function (Odio & Brodish 1990, Dickstein *et al.* 1991, Broide *et al.* 1995).

8.1.2 Basal activity of the HPA axis

A good understanding of the normal variability in baseline HPA axis activity levels is a prerequisite for the use of any physiological or behavioural parameter as an index of stress. Therefore, one of the primary aims of the research was to identify and quantify for the first time in red deer, ultradian, circadian and seasonal rhythms in basal activity of the HPA axis. Remote collection of 24 h blood sample profiles at different times of year revealed a pattern of episodic release of cortisol from the adrenal gland which was consistent with those reported for other species including humans (Krieger *et al.* 1971), primates (Sarnyai *et al.* 1995), sheep (Fulkerson & Tang 1979), cattle (Ladewig & Smidt 1989, Lefcourt *et al.* 1993), horses (Irvine & Alexander 1994) and Eld's deer (Monfort *et al.* 1993). This pattern of release appears to represent a fundamental aspect of HPA axis activity and is a feature common to many other neuroendocrine systems (e.g. the hypothalamic-pituitary-gonadal axis), which is critical for the normal functioning and regulation.

8.1.2.1 Circadian rhythm in HPA axis activity

A circadian rhythm in cortisol secretion was also evident in many of the 24 h baseline profiles obtained at different times of the year. No evidence exists in previous studies of deer for a circadian rhythm in HPA axis activity (white-tailed deer, Bubenik *et al.* 1983; rusa deer, van Mourik & Stelmasiak 1984; Eld's deer, Monfort *et al.* 1993). However, animals in these studies were subjected to either handling or indoor housing, factors known to attenuate circadian rhythms in other species (Irvine & Alexander 1994). The functional significance of this circadian rhythm in cortisol secretion in red deer has yet to be investigated.

However, when present, the circadian rhythm of cortisol secretion in red deer is characterised by an amplitude approximately half that of the corresponding 24 h mean concentration and a variable acrophase that is generally confined to the crepuscular (dawn and dusk) and nocturnal periods of the 24 h cycle. The circadian rhythm in HPA axis activity in other mammals is entrained by the light-dark cycle (Dallman *et al.* 1987b), feeding (Saito *et al.* 1989) and activity/sleep wake cycles (Born *et al.* 1997). Thus, the distribution of acrophase times

observed in red deer may reflect the predominance of crepuscular and nocturnal activity patterns reported for this species (Clutton-Brock *et al.* 1982, Georgii & Schröder 1983, Carranza *et al.* 1991). The variability in timing of the acrophase between experiments may also reflect the effects of varying patterns of behaviour on cortisol secretion. Plasticity in the circadian pattern of behavioural activity has been reported as a prominent feature of red deer behaviour, with shifts in activity patterns occurring in response to external influences such as weather, predation and mating behaviour (Clutton-Brock *et al.* 1982, Georgii & Schröder 1983, Carranza *et al.* 1991).

A number of animals in the present studies failed to exhibit a significant circadian rhythm in cortisol secretion. In deer and ruminants in general maintained at pasture the feeding and activity cues available for entrainment of the circadian rhythm may be weaker than those for non-ruminants or ruminants maintained indoors on fixed feeding schedules. It is well known that ruminants such as deer, sheep and cattle maintained at pasture graze and rest in multiple bouts throughout the day and night (Gates & Hudson 1983, Kilgour & Dalton 1984) which may reduce the strength of the zeitgeber. Simonetta *et al.* (1991) demonstrated that sheep held indoors and feed once a day exhibited a significant circadian rhythm in plasma cortisol whereas sheep fed at multiple times during the day and night showed no significant rhythm in cortisol.

Some form of mild stress associated with the sampling procedure may have also contributed to a lack of circadian rhythmicity in cortisol secretion in some of the animals. In many species stress has been shown to attenuate circadian rhythmicity by increasing the secretion of cortisol during the normal nadir of the rhythm (e.g. horse, Irvine & Alexander 1994). However, in red deer chronic social stress (Chapter 7), failed to attenuate the circadian rhythms of 2 of the 3 animals successfully sampled following mixing. Whether this lack of disruption to the circadian rhythm reflects a physiological difference in the regulation of the HPA axis in red deer or ruminants compared with other species is difficult to ascertain due to the small number of animals sampled in the study. However, it would be interesting in future studies to investigate the effects of different types of stress on the circadian rhythm of HPA axis activity.

8.1.2.2 Seasonal rhythm in HPA axis activity and function

Parameters describing basal activity of the HPA axis displayed a strong seasonal pattern which was remarkably consistent across years. For example, the mean 24 h cortisol concentrations from separate studies conducted in different years were 4.0 ± 1.0 and 4.0 ± 0.8 ng/ml during autumn (Chapters 5 & 6, respectively) and 12.5 ± 1.0 ng/ml and 12.9 ± 1.5 ng/ml during November (Chapters 5 and 7, respectively). The similarity in basal activity of different groups of stags monitored in different years clearly demonstrates that the seasonal cycle in HPA axis activity is a robust phenomenon that can be readily identified using low stress sampling methods.

Androgen dependent mechanisms appear to play an important role in the marked seasonal rhythm in basal cortisol concentrations exhibited by red deer stags (Ingram *et al.* 1999, Chapter 5 & 6). Much of this fluctuation in cortisol levels can be attributed to the seasonal rhythm in adrenal responsiveness to ACTH. This rhythm involves greater plasma cortisol responses to ACTH challenge during the spring/summer period of reproductive quiescence compared with the breeding season and winter months (Cassidy 1996, Suttie *et al.* 1995, Ingram *et al.* 1999, Chapter 5) when endogenous androgen concentrations are elevated (Suttie *et al.* 1992). The mechanism by which androgens are involved in suppressing adrenal function has not been determined for red deer stags, but is likely to involve a direct androgenic suppression of specific steroidogenic enzymes involved in the synthesis of glucocorticoids (Gaskin & Kitay 1971, Hornsby 1982; Miller 1988).

Comparisons of the HPA axis function of castrates during the rut with entire stags during the seasonal period of reproductive quiescence (spring and early summer) suggest that the removal of androgen inhibition of HPA axis activity constitutes only a part of the regulatory changes responsible for the higher basal activity observed in entire stags during spring and early summer. Factors other than androgen removal, involved in the spring and early summer increase in HPA axis activity have yet to be elucidated for red deer, however variations in metabolic demand, fat accumulation and growth of velvet antler may all contribute.

There appears to be a compensatory response of the cortisol binding capacity of plasma to changes in total cortisol concentrations during the rut and spring summer period. In entire stags monitored during the rut (Chapter 6), the proportion of free cortisol in plasma during basal conditions (12.9 ± 0.9 %) was greater than that observed in entire stags during the nadir of reproductive function (9.8 ± 0.6 %). In contrast, concentrations of free cortisol in entire stags during the rut (0.41 ± 0.2 ng/ml, Chapter 6) were comparable to those measured in entire stags in November (0.32 ± 0.10 ng/ml, Chapter 7). Males of other seasonally breeding species exhibit an androgen dependent fall in CBG concentration, and hence plasma cortisol binding capacity, during the breeding season. However, in these species, changes in plasma cortisol binding capacity are generally associated with an increase in unbound cortisol concentrations resulting in increased bioavailability of glucocorticoids (McDonald *et al.* 1981, Bradley & Stoddard 1992). In contrast, the change in cortisol plasma binding seen in red deer stags appears to represent a homeostatic mechanism designed to maintain the bioavailability of cortisol within tightly regulated limits throughout the seasonal nadir (rut) and peak (spring/summer) of endogenous HPA axis activity.

There was no parallel seasonal change observed in pituitary function, with the peak cortisol response to CRH challenge being similar in stags both during the rut (15.9 ± 5.8 ng/ml) and in spring and early summer (15.8 ± 2.2 ng/ml) periods. This would suggest that sites other than the anterior pituitary (e.g. PVN) may contribute to the increased activity of the HPA axis an spring and summer.

8.1.3 The HPA axis response to acute stress

In the assessment of stress, an essential requirement for any physiological or behavioural index is its ability to differentiate between stressors of varying intensity. The studies presented in this thesis have concentrated on the measurement of total cortisol as an index of acute stress in red deer stags. Using the DracPac system, significant elevations in plasma cortisol concentrations were obtained in response to a variety of acute stressors including yarding, restraint in a pneumatic deer crush, manual blood sampling and mixing with unfamiliar conspecifics. These responses were similar to other routine handling procedures previously evaluated

using the DracPac technique, such as restraint (Carragher *et al.* 1997) removal of growing antler (Matthews *et al.* 1994) and transport (Waas *et al.* 1997, 1999). In these acute stress situations, increased plasma cortisol responses are apparently associated with an increase in intensity of handling (Carragher *et al.* 1997), and may reflect the animal's perception of the severity of the stressor imposed. However, this relationship may not extend to the differentiation between more severe types of stressors where the magnitude of the HPA axis response may be limited, at least in amplitude, by the capacity of the adrenal to synthesise cortisol. A plateau in adrenal response to increasing doses of ACTH was observed (see Chapter 4), though the duration of the response continued to increase. Therefore, when assessing severe acute stressors it may be better to use measures such as area under the adrenal response curve which does not exhibit this plateau effect. However, it must be emphasised that whatever the HPA axis parameter measured, it is important that a low stress blood sampling technique is used and that frequency and duration of blood sampling be sufficient to fully characterise the response, tasks for which the DracPac system is ideally suited for.

In many situations (e.g. quantifying the effects of an acute stressor at different times of year) the measurement of total plasma cortisol concentrations may not be appropriate due to seasonal changes in basal and stress induced total cortisol concentrations. In such cases, other measures of HPA axis activity (e.g. levels of unbound cortisol) offer distinct advantages since both baseline and ACTH induced maximum concentrations of unbound cortisol appear to remain consistent over the year. This observation is likely to reflect the tight regulatory control of unbound cortisol exhibited by red deer.

8.1.4 The HPA axis response to chronic or repeated stress

Compared with acute stress, the response of the HPA axis to chronic or repeated stress is not well understood. With continued exposure to chronic stress, glucocorticoid concentrations may remain elevated (Roman-Ponce et al 1981), normalise (Ladewig & Smidt 1989), or become depressed (Rhynes & Ewing 1973). In deer subjected to chronic social stress (Chapter 7), the initial response to mixing involved activation of the HPA axis. However, long term changes in

HPA axis activity and function were characterised by a lowering of basal cortisol concentrations (hypocortisolemia), an increase in the proportion of unbound cortisol (although free cortisol concentrations did not change) and a reduction in the responsiveness of the adrenal to ACTH and the pituitary to CRH.

Stress has been associated with activation of the HPA axis and increased cortisol secretion to such an extent, that stress and increased cortisol secretion have become synonymous in the literature (Heim *et al.* 2000). However, it is clear from this study and other reports of the response of red deer to chronic stress (Goddard *et al.* 1994, 1996, Hanlon *et al.* 1994, 1995, Pollard & Littlejohn 1998) that chronic stress is often associated with reductions in basal cortisol concentrations (hypocortisolemia). Therefore, measures of cortisol secretion commonly used in the assessment of acute stress may not be applicable to the assessment of chronic stressors.

There is increasing evidence that hypocortisolism maybe a more prevalent response to chronic stress than previously thought (reviewed in Heim *et al.* 2000). Factors which may contribute to the development of either hypercortisolism or hypocortisolism include differences in quantitative and qualitative properties of the stressor, specific individual and species differences, and differences in experimental protocols, including differing sampling times and methods. In addition, Jensen *et al.* (1996a) proposed that rather than being a permanent state, the response to chronic stress may change over the time course of exposure to the stressor. These changes are characterised by an initial activation of the HPA axis followed by biochemical adjustments that result in an apparent normalisation or even suppression of the activity of the HPA axis over time (Jensen *et al.* 1996a). The rate at which these changes occur are dependent on characteristics of the stressor such as stressor severity and frequency of exposure, and individual animal factors such as the animals perception of controllability, predictability and monotony of the stressor (Jensen *et al.* 1996a). Thus, the measured effects of chronic stress on the HPA axis could be influenced by both the rate of adaptation and the stage during the process when the axis is evaluated. Figure 8.1 depicts a model hypothesis based on that of Jensen *et al.* (1996a) to account for the changes in total cortisol concentrations of deer subjected to repeated mixing into novel

social groups. The initial activation of the HPA axis and elevation in basal cortisol concentrations is followed by a process of normalisation and eventually suppression of basal HPA axis activity. The duration of this basal cortisol suppression following the removal of the stressor has yet to be determined in deer.

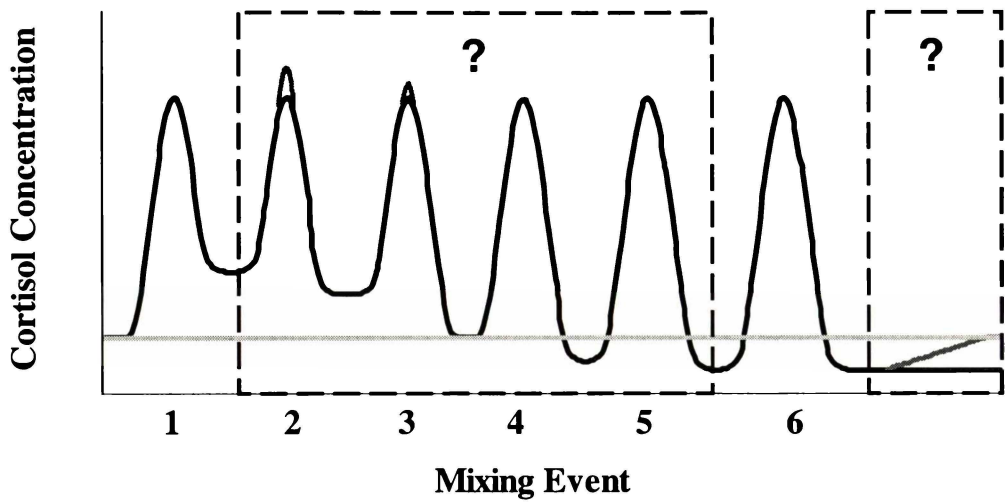


Figure 8.1 A proposed model of the HPA axis response to repeated mixing in the red deer stag. The light grey horizontal line represents the pre-mixing 24 h mean baseline. The dark grey lines on top of mix 2 and 3 represent possible increased responsiveness of the HPA axis. The black line represents the total cortisol response to each mixing event. The curves within the dashed boxes represent the predicted changes in total cortisol.

Chronic social stress (Chapter 7) produced a reduction in adrenal responsiveness to ACTH challenge in the red deer stag. In red deer, the adrenocortical response to ACTH has been reported to increase (Hanlon *et al.* 1995), remain unchanged (Hanlon *et al.* 1994) or to decline (Goddard *et al.* 1994, Pollard & Littlejohn 1998) relative to baseline concentrations during chronic social stress. In other species, quantifying the responsiveness of the adrenal cortex by exogenous ACTH challenge following chronic stress, has also produced conflicting results. For example in cattle, heat stress (Roman-Ponce *et al.* 1981), and tethering (Ladewig & Smidt 1989) result in a reduced adrenal response to ACTH, whereas chronic social stress and competition for free stalls caused an enhanced adrenal response (Friend *et al.* 1977, 1979). These differences in response to ACTH can also show

transient changes in response to chronic stress returning to pre-stress levels over time (Jensen *et al.* 1996a). Thus, as a index of chronic stress the adrenal response to ACTH may suffer many of the same problems in interpretation that measurements of cortisol secretion have.

A reduction in the responsiveness of the pituitary to CRH was also seen in red deer subjected to chronic social stress. Pituitary responsiveness to CRH has not previously been assessed in red deer, however, a reduction in pituitary responsiveness (ACTH secretion) to exogenous CRH challenge following chronic social stress has been previously reported in horses (Alexander *et al.* 1996). Reduced pituitary responsiveness to CRH is also characteristic of a number of human psychiatric disorders such as depression, panic disorder, anorexia nervosa and post-traumatic stress disorder (Smith *et al.* 1989). As an index of chronic stress, tests of pituitary function using CRH challenges may offer a number of advantages over the more traditional adrenal function test. The relative magnitude of the changes seen in pituitary function during chronic stress were greater than the changes in adrenal function. In addition, pituitary responsiveness was not affected by the seasonal changes in other components of the HPA axis.

Chronic stress can also lower the cortisol binding capacity of plasma, thereby increasing the proportion of biologically active free cortisol present in circulation (Alexander & Irvine 1998, Blanchard *et al.*1993, Kattesh *et al.* 1980, Spencer *et al.* 1996, Chapter 7). The function of this stress-induced fall in binding capacity is unclear, but in some species, the resulting increased free glucocorticoid concentrations may have a greater effect on target tissues (Fleshner *et al.* 1995). In the red deer, free cortisol concentrations were similar prior to and following mixing and between different times of year. The maintenance of baseline free cortisol concentrations within a narrow band appears to be highly regulated in the red deer stag. Thus, perturbations to these levels may signify severe stress and the progression to a pre-pathological state in red deer. The cortisol binding capacity of plasma may also offer some potential as a measure of chronic stress in deer. The main determinant of this binding CBG has a relatively long half-life in plasma. Therefore, CBG concentrations may offer a more robust measure of chronic stress effects on the HPA axis which is unaffected by the acute handing

stress required to obtain manual blood samples. However, in deer a seasonal rhythm is also apparent in cortisol binding capacity of plasma which may complicate the interpretation of stress induced changes.

In the red deer stag, chronic stress is characterised by an acute period of increased basal HPA axis activity followed by an adaptive biochemical down-regulation at different levels of the HPA axis. These changes may function to protect the organism from detrimental effects on growth, reproduction and immune status due to sustained high levels of glucocorticoids. However, changes in cortisol binding capacity of plasma may provide a mechanism maintaining baseline HPA axis function during chronic social stress in the stag.

8.1.5 Conclusion

In summary, a number of measures of HPA axis activity and function change during acute or chronic stress and therefore appear to offer opportunities for quantifiable measurement of the stress response in red deer stags. However, many of these measures exhibit endogenous rhythms in activity (e.g. ultradian, circadian and seasonal) or adaptations to stress which complicate interpretation. These measures of HPA axis activity and function are also readily confounded by the stress associated with handling necessitating the use of stress free monitoring techniques. The DracPac remote blood sampling device offers such a stress-free technique for obtaining undisturbed measurement of HPA axis activity and function without the confounding effects of repeated handling. However, even with such powerful techniques, it is essential that HPA axis responses be interpreted in context with changes in other physiological and behavioural measures of stress.

APPENDIX 1

EXTRACTED RADIOIMMUNOASSAY FOR CORTISOL IN RED DEER PLASMA

Introduction:

Plasma cortisol concentrations were determined in duplicate, after extraction in ethyl acetate, by an ^{125}I radioimmunoassay method with polyethylene glycol separation. A standard curve, using charcoal stripped deer plasma (for stripping method see Chard 1995), spiked with cortisol to produce concentrations of 0, 1, 2, 5, 10, 20, 50, 100 ng/ml was used to determine the concentration of cortisol in individual plasma samples. The inter and intra-assay co-efficient of variations were determined using spiked deer plasma controls of known low (5 ng/ml), medium (20 ng/ml) and high (50 ng/ml) cortisol concentrations.

Standard Curve (assayed in quadruplicate):

TC (total counts)

NSB (non specific binding)

Standards 0, 1, 2, 5, 10, 20, 50, 100 ng/ml cortisol in charcoal stripped deer plasma (Chard, 1995).

Controls (assayed in duplicate every 50 samples):

Red deer plasma spiked with cortisol to produce plasma of known low (5 ng/ml), medium (20 ng/ml) and high (50 ng/ml) cortisol concentrations.

Samples (assayed in duplicate)

Procedure

Extraction:

- Add 25 µl of standards, controls and samples to glass or polypropylene tubes.
- Add 1 ml of redistilled Ethyl Acetate, Shake for 1 minute, allow to settle.
- Aliquot by pipette 100 µl of the Ethyl Acetate into duplicate glass or polypropylene tubes making sure not to collect the denatured protein at the bottom of the tube.
- Dry down in fume hood, at this point evaporated samples can be stored in the fridge for several days.

Reconstitution:

- When solvent has evaporated entirely reconstitute with 100 µl of Bovine γ Globulin (1% in PBS gel).

Assay:

- Add 100 µl of Antisera (75000:1 in PBS gel), shake for 1 minute and incubate at room temperature for 30 minutes.
- Add 100 µl of cortisol ^{125}I tracer (15000 cpm), shake for 1 minute and incubate at room temperature for 30 minutes.
- Incubate overnight at 4°C (approximately 16 h).
- Next morning add 1 ml of 20% PEG 6000 chilled to 4 °C, shake for 1 minute.
- Centrifuge at 2400 rpm for 20 minutes at 4 °C.
- Tip off supernatant and count precipitate in Gamma counter.

Solutions

PBS buffer stock: 0.5 mol/l sodium phosphate buffer, pH 7.0.

(dilute 5x for use) 4.5 % NaCl

0.5 % NaN_3

To make PBS buffer stock 1l:

dissolve	109.1 g	$Na_2HPO_4.12H_2O$	or 54.2 g	$Na_2HPO_4.2H_2O$
	30.4 g	$NaH_2PO_4.12H_2O$		
	45.0 g	NaCl		
	5.0 g	NaN_3		
in	1 l	milli Q distilled H_2O		

PBS gel buffer:

Dilute stock PBS buffer 5x in distilled water.

Add 0.1% (ie. 1 g/l) gelatine. Buffer must be heated to dissolve gelatine.

1% Bovine γ Globulin:

Dissolve 1 g Bovine γ Globulin in 100 ml of PBS gel (lasts approx. 1 week)

Antisera (R1) (developed at Ruakura):

Cortisol antisera (R1) is stored frozen at 1:100 dilution. Dilute by a further 750 x in PBS gel to give a final dilution of 75000:1. (Make fresh each day).

Tracer:

Cortisol-3-(*O*-carboxymethyl) oximino- (2-[^{125}I]iodohistamine) (Amersham Pharmacia Biotech, Auckland, NZ). Dilute stock tracer with PBS gel to give 15000 cpm per 100 µl.

20% PEG:

200 g/l of polyethylene glycol 6000 dissolved in distilled water. PEG must be cooled to 4°C before using.

Cross Reactivity of Cortisol Antisera

Dexamethasone	0.02
17α OH Progesterone	0.10
Cholesterol	<0.005
$E_2 17\beta$	<0.005
Oesterone	<0.005
Androstedione	<0.005
Testosterone	0.007
Androstenediol	<.005
Epitestosterone	<.005
Corticosterone	0.82
11Deoxy 17OH Corticosterone	5.70
Deoxycorticosterone	0.005
Cortisone	1.24

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