Corticosterone responses of captive and wild northern brown kiwi (*Apteryx mantelli*)

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Abstract

Conservation strategies should work to minimise the occurrence of stressful situations, which are likely to result in chronic elevations of corticosterone. Consequently, identification of such situations would yield important information for conservation management. The objective of this study was to compare the basal levels of corticosterone and the corticosterone response of kiwi (*Apteryx mantelli*) in different management systems. Repeated blood sampling enabled us to describe the magnitude and duration of the corticosterone response, which is a measure of the sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis to stress. All kiwi responded to capture and handling with a rise in plasma levels of corticosterone, which peaked 30 min after capture. Corticosterone levels immediately after capture in wild kiwi were significantly higher than those of captive kiwi. This was most likely due to the method of capture, as wild kiwi took considerably longer to catch than captive kiwi. The plasma level of corticosterone 30 min after capture in nocturnal house kiwi was significantly lower than wild kiwi, but similar to those in outdoor penned kiwi. The cause of these differences is unclear. Nocturnal house kiwi may have become habituated to the presence of humans. Alternatively, it may be due to nocturnal house kiwi being held on a different light cycle to outdoor pen and wild kiwi. In addition, corticosterone levels in nocturnal house kiwi returned to basal levels 2 h after capture and handling. There was no significant difference in the binding affinity (K_d) and binding capacity (B_{max}) of corticosteroid binding globulin (CBG) between nocturnal house, outdoor pen and wild kiwi. Furthermore, at no stage did maximum plasma levels of corticosterone exceed B_{max}. Therefore, the B_{max} of kiwi CBG was not a major factor when interpreting corticosterone responses of kiwi. Regularly handled kiwi do not respond to public display and handling with an increase in plasma levels of corticosterone. Therefore, these kiwi appear to have become habituated to this procedure. Elevated corticosterone levels in wild kiwi immediately after capture indicate that determining the precise location of wild kiwi is sufficient to induce a stress response. Therefore this practice should be kept to a minimum. Low basal levels of corticosterone indicate that captive kiwi have acclimatised to captivity. Furthermore, these results indicate that captive kiwi are not exposed to chronic elevations in corticosterone.

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1 General introduction

1.1 The avian stress response

Animals regularly encounter both internal and external stimuli that threaten homeostasis (stressors) (Harvey et al., 1984). In response to these suddenly threatening events, many behavioural and physiological changes occur that serve to restore homeostasis, and to enhance survival and long term reproductive success (stress response)(Carsia and Harvey, 2000). These can be specific to the particular stressor involved. For example, during increased ambient temperature birds may dilate surface blood vessels and sweat to increase heat dissipation (physiological response), and seek out cool surfaces (behavioural response) (Siegel, 1995). Birds may also respond in a nonspecific manner, regardless of the stressor involved (e.g. heat, cold, predator, storm, (Siegel, 1995)).

Characteristic of the nonspecific stress response is the initial activation of the sympathetic adrenomedullary system (SAS), followed soon after by activation of the hypothalamic pituitary adrenocortical (HPA) axis (Siegel, 1980; Harvey et al., 1984). Stimulation of the SAS constitutes the 'alarm' reaction, characterised by sudden increases in catecholamines within seconds of encountering the stressor (reviewed in Siegel, 1995; Carsia and Harvey, 2000). The resulting physiological responses include increases in blood pressure, respiration rate, muscle tone and blood sugar levels (Siegel, 1995). Although activation of the HPA axis occurs simultaneously with activation of the SAS, its effects (release of corticosteroids) are not discernible until several minutes later (Siegel, 1980). It is now generally accepted that the release of corticosterone, due to HPA axis stimulation by a stressor, is the distinguishing feature of the nonspecific stress response in birds (Carsia and Harvey, 2000).

1.2 Endocrine control of the hypothalamic pituitary adrenal (HPA) axis in birds

1.2.1 Control of corticotropin releasing factor (CRF)

Humoral and neural stimuli can impinge on the hypothalamus to alter hypothalamic neurotransmission (Harvey et al., 1984). This can result in the release of corticotropin releasing factor (CRF) from hypothalamic peptidergic neurones into the portal blood system (Harvey et al., 1984). When CRF reaches the anterior pituitary, it stimulates the release of adrenocorticotrophic hormone (ACTH) (Carsia and Harvey, 2000).

A variety of nervous and endocrine inputs can vary the synthesis and release of CRF in mammals. For example, an afferent nerve supply allows visceral and somatic sensory input to impinge on CRF secretion (Chadwick et al., 1993). In addition, such substances as serotonin, interleukins 1 and 6, cytokines and acetylcholine can affect CRF gene expression (Chadwick et al., 1993). In the rat, synthesis and release of CRF can also be suppressed by stress-induced increases in glucocorticoids. This is caused by the down regulation of corticosterone receptors in the hypothalamus (Sapolsky et al., 1984). Adrenaline has also been shown to regulate the activity of CRF neurones in mammals (Axelrod and Reisine, 1984). Similar negative feedback mechanisms may also be involved in avian species (Harvey et al., 1984).

1.2.2 Control of adrenocorticotrophic hormone (ACTH)

In response to various endocrine stimuli, ACTH is released from the cephalic lobe of the anterior pituitary after a bird has encountered a noxious stimulus (stressor) (Carsia and Harvey, 2000). ACTH is released into the systemic circulation of birds, where it stimulates the release of glucocorticoids from the adrenal gland (Harvey et al., 1984). Although the principal ACTH secretagog is CRF, other hypothalamic peptides, catecholamines (adrenalin, noradrenaline, Carsia and Harvey, 2000) and indoleamines may evoke ACTH release (Harvey et al., 1984). The strongest of these is arginine vasotocin (AVT), which has a distribution similar to CRF in the median eminence of

some species (Norris, 1997; Carsia and Harvey, 2000). Whether CRF or AVT is the primary ACTH secretagog is dependent on the type of stressor involved (Carsia and Harvey, 2000). Furthermore, whether CRF or AVT are released in redpolls (*Carduelis flammea*) depends on the physiological status of the bird (Romero et al., 1998d), suggesting a system similar to that seen in rats (Axelrod and Reisine, 1984). Therefore, under different ecological and physiological conditions the primary ACTH secretagog (CRF or AVT) may change (Carsia and Harvey, 2000). A synergism between vasopressin, α-adrenergic agonists and synthetic CRF for the release of ACTH has been demonstrated in rats (Axelrod and Reisine, 1984), but not yet in birds (Harvey et al., 1984; Carsia and Harvey, 2000). Although its effect *in vivo* is inconsistent, mesotocin also appears to be an avian ACTH secretagog (Carsia and Harvey, 2000).

Various compounds have been shown to prevent the release of ACTH from the pituitary gland. For example, both somatostatin and the opioids can inhibit the release of ACTH in birds (Carsia and Harvey, 2000). This also occurs in rats, where somatostatin blocks ACTH release by inhibiting adenylate cyclase activity (Axelrod and Reisine, 1984). A similar mechanism may also underlie the ability of somatostatin to inhibit ACTH release in birds. In addition, glucocorticoids are known to inhibit the release of ACTH and ACTH-like peptides (Harvey et al., 1984). In mammals, this can occur by a reduction in mRNA activity or by affecting cAMP-stimulated ACTH release (Axelrod and Reisine, 1984). Again, a similar mechanism probably underlies these inhibitory effects of glucocorticoids on avian ACTH release.

1.2.3 Control of corticosterone release

Corticosterone is the primary glucocorticoid released when the avian HPA axis is stimulated (Carsia and Harvey, 2000). It is generally accepted that ACTH is the primary secretagog of corticosterone (Carsia and Harvey, 2000). The release of ACTH into peripheral circulation increases the rate of release and biosynthesis of corticosterone from adrenal cortical cells (Harvey et al., 1984), often coinciding with adrenal hypertrophy (Carsia and Harvey, 2000). As with the hypothalamus and pituitary, other circulating hormones acting at the adrenal can cause either negative feedback on corticosterone release, or act as a corticosterone secretagog.

Glucocorticoids, particularly corticosterone, appear to exert a negative feedback influence at all levels of the HPA axis (Carsia and Harvey, 2000). For example, corticosterone can decrease the adrenal responsiveness to ACTH (Harvey et al., 1984). However, stress-induced rises in ACTH secretion have been shown to override this inhibitory feedback mechanism of corticosterone (Harvey et al., 1984). Catecholamines (dopamine, adrenaline, noradrenaline) and acetylcholine can repress basal corticosterone release but not stress-induced levels (via activation of α-adrenergic receptors). Conversely, catecholamine activation of β-adrenergic receptors can in fact augment the adrenocortical response to endogenous ACTH (Carsia and Harvey, 2000). In general though, catecholamines seem to have a positive effect on adrenocortical cells, promoting the release of corticosterone. A similar positive regulation of adrenocortical function has been demonstrated by prolactin, parathyroid hormone, vasoactive intestinal peptide (VIP), and opioids (Carsia and Harvey, 2000).

1.2.3.1 Environmental factors influencing corticosterone release

The release of corticosterone from the adrenal gland can be influenced by environmental factors such as season (e.g. breeding, moult and migration), time of day, and food quality and availability. The endocrine bases for this control is often complex, involving several levels of the HPA axis at once. For example, during moult (an energetically costly event), regression of the reproductive system of redpolls is associated with a decrease in adrenocortical function (Romero et al., 1998d). Romero et al. (1998c) demonstrated that this decrease in adrenocortical function is not caused by adrenal insensitivity to ACTH (since exogenous ACTH caused corticosterone release), but was due to (a) a reduction in CNS signals and hypothalamic capacity to secrete CRF and AVT, (b) a reduced pituitary response to these hypothalamic signals, and (c) a reduced adrenal capacity to secrete corticosterone (Romero et al., 1998b; Romero et al., 1998d).

In some species, breeding season is associated with an increase in circulating levels of corticosterone, and the corticosterone response. For example, in breeding white-crowned sparrows (*Zonotrichia leucophrys gambelii*), reduced negative feedback on the

HPA axis by corticosterone (Astheimer et al., 1994), and an enhanced adrenocortical response to ACTH (Romero and Wingfield, 1998), results in an increase in plasma corticosterone levels. However, in other species corticosterone responses are lower during the breeding season (e.g. male pied flycatchers (*Ficedula hypoleuca*) (Silverin, 1998a; Silverin and Wingfield, 1998), willow tit (*Parus montanus*) (Silverin, 1998b) and Inca dove (*Scardafella inca*) (Wingfield et al., 1992)).

A diurnal rhythm in basal levels of corticosterone is seen in some birds, with levels peaking at dusk and dawn (reviewed in Carsia and Harvey, 2000). Variations in the activity of ACTH, CRF and hypothalamic aminergic neurones are presumed to control the rhythm (Carsia and Harvey, 2000). Recent evidence also suggests that there is a diurnal rhythm in the sensitivity of the HPA axis, with the greatest adrenocortical response to stress occurring at the beginning of the active period (Breuner et al., 1999).

The nutritional status of a bird can also affect adrenocortical activity (Carsia and Harvey, 2000). For example, food or protein restriction (of sufficient duration) typically causes an increase in plasma levels of corticosterone (see Harvey et al., 1984; Carsia and Harvey, 2000). Although ACTH concentrations are depressed during protein restriction, there is an increase in adrenal weight, cellular steroidogenic capacity, corticosteroidogenic responsiveness and sensitivity to ACTH (Carsia et al., 1988; Carsia and Harvey, 2000). There is also an increase in the affinity and concentration of cellular ACTH receptors (Carsia and Weber, 1988).

1.2.3.2 Human factors influencing corticosterone release

Regular association with humans may also influence the sensitivity of the HPA axis to stressors. For example, in Magellanic penguins (*Spheniscus magellanicus*) and chickens (*Gallus domesticus*) regular association with humans can result in lower corticosterone levels and a reduced fear of humans (Jones, 1993; Fowler, 1999). There are also many examples in birds of regular handling by humans reducing the bird's adrenocortical response to further handling (e.g. Barnett et al., 1994; Hemsworth et al., 1994; Kannan and Mench, 1996; Collette et al., 2000). The mechanisms underlying this apparent habituation to humans is poorly understood. One hypothesis is that the

reduced response is caused by increasing the sensory threshold required to elicit a response (psychological coping) (Harvey et al., 1984). It is possible then that due to prior experience (expectation of events) the handling stimulus is no longer deemed stressful by the individual. However, the avian adrenal gland is capable of becoming desensitised to high and prolonged treatments of exogenous ACTH (Kalliecharan, 1981; Davison et al., 1985). Therefore, any apparent reduction in corticosterone levels might be due to a down regulation in the adrenocortical responsiveness to corticosterone secretagogs, rather than to a change in the animals perception of the stimulus.

1.2.4 Corticosterone binding globulin (CBG)

The concentration of circulating corticosterone is dependent on its rate of secretion, uptake by tissues and metabolic clearance by the liver and kidney (Wingfield et al., 1984). However, approximately 80 – 90% of plasma corticosterone circulates bound to a specific protein, corticosteroid binding globulin (CBG) (Westphal, 1969). In general, only unbound (free) corticosterone can enter cells (Thompson and Lippman, 1974). In addition, CBG bound corticosterone is cleared less quickly from the circulation than the free fraction (Siiteri et al., 1982). Therefore, the binding capacity and affinity of CBG in the plasma is important in determining the amount of 'physiologically active' (free) corticosterone. The circulating concentrations of CBG can vary with sex (Siiteri et al., 1982; Silverin, 1986), season (Silverin, 1986; Klukowski et al., 1997; Romero et al., 1998b; Romero and Wingfield, 1998) and time of day (Siegel et al., 1976; Meier et al., 1978; Kovacs and Peczely, 1983). Therefore, characterisation of the binding capacity, affinity and specificity of CBG in plasma is necessary to correctly interpret corticosterone responses of birds.

1.3 Consequences of elevated corticosterone levels

The perception of a stressor typically causes a rise in plasma corticosterone levels in birds (Carsia and Harvey, 2000). A rise in corticosterone levels in the short term (minutes to hours) can be beneficial to the animal, promoting behaviours and physiological changes that aid immediate survival and long term reproductive success.

However, if corticosterone levels remain elevated for longer periods (days to weeks), then homeostasis may not be restored, resulting in the 'exhaustion' phase (Selye, 1937) and its attendant deleterious effects (Wingfield et al., 1998). These responses will be discussed briefly.

1.3.1 Short term elevation of corticosterone

A primary role of corticosterone is the promotion of gluconeogenesis, the formation of glucose from protein and fat (reviewed in Harvey et al., 1984; Wingfield et al., 1998; Carsia and Harvey, 2000). Elevated plasma levels of corticosterone may also promote foraging behaviour (Wingfield et al., 1998). For example, corticosterone implants led to a non-significant increase in foraging by white-crowned sparrows and song sparrows (*Melospiza melodia*) (Astheimer et al., 1992). In addition, replacement therapy with corticosterone in metyrapone treated white-crowned sparrows increased foraging (Wingfield et al., 1998). Corticosterone administration also increased the rate of food intake in the domestic chicken (Nasir et al., 1999). It would appear then, that through gluconeogenesis and increasing the rate of food intake, corticosterone plays an important role in increasing the energy available during stressful events (e.g. avoiding a predator, or during a storm).

Elevated levels of corticosterone can inhibit reproductive and territorial behaviour (Wingfield et al., 1998) whilst maintaining the reproductive system in a near functional state (Wingfield and Silverin, 1986; Wingfield et al., 1997). For example, implants of corticosterone reduced parental behaviour (feeding of young and nest abandonment) in pied flycatchers (Silverin, 1986), and territorial aggression in male song sparrows (Wingfield and Silverin, 1986). Similarly, in male roughskin newts (*Taricha granulosa*) administration of corticosterone rapidly blocked courtship clasping of females (Rose et al., 1993). Transitory changes in behaviour such as this enable the animal to cope with the immediate stressor, while still maintaining the ability to quickly return to breeding should conditions improve.

Acute rises in corticosterone may also promote diurnal escape behaviour (Wingfield et al., 1998). For example, corticosterone treatment of male white-crowned sparrows

caused an increase in perch hopping activity during periods of food shortage (Astheimer et al., 1992), which may encourage movement away from the stressor (reviewed in Wingfield et al., 1998). Nocturnal restfulness in birds may also be associated with short term rises in corticosterone (Wingfield et al., 1998). For example, corticosterone treatment apparently eliminates episodes of increased oxygen consumption, resulting in energy savings during the night (Buttemer et al., 1991 cited in Wingfield et al., 1998).

1.3.2 Long term elevation of corticosterone

It is generally accepted that chronically elevated levels of corticosterone can inhibit the reproductive system of birds (see Silverin, 1998b; Wingfield et al., 1998; Carsia and Harvey, 2000). Elevated levels of corticosterone can cause a decline in the concentrations of hormones associated with reproduction (e.g. gonadal steroids), thereby inhibiting gonadal function (reviewed in Silverin, 1998b and Carsia and Harvey, 2000). However, short periods of elevated corticosterone levels do not necessarily depress luteinizing hormone (LH) and testosterone (reviewed in Silverin, 1998b).

Chronically elevated levels of corticosterone may also inhibit the immune system (reviewed in Siegel, 1980; Carsia and Harvey, 2000). This may not be surprising since cells of the immune system are known to have glucocorticoid receptors (Carsia and Harvey, 2000). Long term elevations of corticosterone are known to cause regression of the lymphoid organs (e.g. thymus, spleen, bursa of Fabricius), depletion of lymphocytes from germinal centres and depression in the number of circulating lymphoid cells (lymphocytes) (Siegel, 1980; Carsia and Harvey, 2000). This can result in a reduced resistance to viral diseases. Conversely, elevated levels of corticosterone, either due to stressors or to corticosterone treatment, can increase the proportion of heterophils, resulting in increased resistance to bacterial disease (Carsia and Harvey, 2000). Given that heterophil/lymphocyte (H/L) ratios rise when corticosterone levels increase, it is not surprising that H/L ratios are increasingly being used as an index of stress (e.g. Vleck et al., 2000).

Glucocorticoids promote gluconeogenesis, which is of benefit to the animal if corticosterone concentrations are elevated for short periods. However, if plasma levels of corticosterone remain elevated for long periods, then there can be a dramatic decrease in body weight and growth (e.g. Saadoun et al., 1987; Hayashi et al., 1994). This is primarily caused by an increase in muscle protein catabolism (Siegel, 1980; Saadoun et al., 1987; Takahashi et al., 1993; Hayashi et al., 1994). The primary stores of protein in birds are the large flight muscles. Excessive loss of protein from these stores will obviously have detrimental effects on the survival and reproductive success of the individual.

1.4 The capture stress protocol

Plasma corticosterone levels in birds usually rise within minutes following exposure to a stressful stimulus (Wingfield et al., 1998; Carsia and Harvey, 2000). Corticosterone levels usually remain elevated for 30 – 60 min, before declining to levels similar to those prior to the stressful event (basal levels) (Wingfield et al., 1992; Wingfield et al., 1997; Wingfield et al., 1998). It is possible to describe the magnitude and duration of the corticosterone response by applying a standardised stressor (capture and handling) and removing blood samples at regular intervals (Wingfield, 1994). This can provide important information regarding the sensitivity of the HPA axis to stress. Therefore, this technique could potentially be used to identify groups of birds that have an increased susceptibility to a stressor (Wingfield et al., 1997).

The application of this technique to captive and wild birds has identified populations whose sensitivity to stress can vary throughout the year. Furthermore, possible ecological and physiological causes of this variation have been identified (Wingfield et al., 1995b). For example, changes in the sensitivity of the HPA axis are associated with breeding (Wingfield et al., 1992; Silverin et al., 1997), moult (Astheimer et al., 1994), establishment of breeding territories (Silverin, 1998a) and migration (Rehder et al., 1986). A decline in the corticosterone response can also occur via regular association with, or handling by, humans (Fowler, 1999; Collette et al., 2000).

1.4.1 The capture stress protocol and the management of kiwi

Conservation strategies should work to minimise the occurrence of stressful events or situations that are likely to result in chronically elevated levels of corticosterone. The likelihood of such events will depend on the sensitivity of the HPA axis to stress (Wingfield et al., 1997). Analysis of the adrenocortical response to stress offers a technique that could potentially identify populations of captive kiwi (*Apteryx mantelli*) with increased sensitivity of the HPA axis.

In New Zealand, kiwi are held in captivity for both advocacy (conservation education) and for captive breeding (see text box below). The effects of these various management systems on the stress response of kiwi are unknown. The objective of this study was to compare the basal levels of corticosterone and the corticosterone response of kiwi in different management systems (see text box below). In doing so, we may be able to identify those captive management systems where kiwi have an increased sensitivity of the HPA axis. Identification of such situations would provide crucial information relevant to the management of kiwi.

Nocturnal house: A floor to sealing glass wall separates the public from the kiwi. The light cycle is reversed so that the public can view active kiwi during the day.

Willowbank: A second type of nocturnal house enclosure where the public are separated from the kiwi by a small wooden fence. The light cycle is reversed so that the public can view active kiwi during the day.

Outdoor pen: Used primarily for captive breeding, these enclosures are secluded, and positioned well away from the public to avoid disturbance.

Outdoor pen-public access: Used primarily for captive breeding, these enclosures allow public access close to the pens. Some institutes also allow night-time tours of the pens.

Regularly handled kiwi: These are kiwi that are brought out of their enclosures and handled regularly for display to the public. A typical display session would involve the removal of the bird from its enclosure and placing it on the ground (or in an arena) within very close proximity to unfamiliar humans for periods up to half an hour.

Wild: A wild population of kiwi at Aotuhia, Taranaki, with individuals that have previously been caught and radio-tagged.

1.5 Outline of thesis

The aim of this study was to determine the sensitivity of the HPA axis (measured as circulating corticosterone levels) of kiwi in different management systems. This thesis consists of five chapters; a general introduction, three experimental studies and a general discussion. The aim of the first experiment (chapter 2) was to assess whether the capture stress protocol was a suitable and practical method for testing the sensitivity of the HPA axis in kiwi. The second experiment (chapter 3) uses the capture stress protocol to compare the corticosterone responses of kiwi in different captive management systems and the wild. The fourth chapter uses quail to validate a method for measuring CBG. A modified protocol was then used to define the binding capacity and affinity of CBG in kiwi plasma. The fifth chapter discusses the major findings of the experimental chapters and how the results relate to the management of kiwi, and provides direction for future research.

2 Pilot study

2.1 Abstract

This chapter presents the results of a pilot study aimed at assessing whether the capture stress paradigm is a suitable method for testing the sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis of kiwi. All six kiwi responded to capture and handling with an increase in plasma levels of corticosterone. These levels peaked (but not significantly so) at 30 min (nocturnal house 327.81 ± 124.19 ng/ml, outdoor pen 233.09 ± 65.10 ng/ml) and remained elevated for 60 min (nocturnal house 393.93 ± 77.94 ng/ml, outdoor pen 221.04 ± 64.91 ng/ml). Corticosterone levels did not differ significantly between nocturnal house and outdoor penned kiwi. Furthermore, gender had no effect on the corticosterone response. Initial plasma levels of corticosterone in kiwi were high (nocturnal house 85.26 ± 52.47 ng/ml, outdoor pen 70.08 ± 31.32 ng/ml). The lack of a significant effect of time on the corticosterone response may have been due to small sample size or to the long (up to 10 min) time taken to obtain each initial blood sample. Consequently, during all further experimentation, each bird would be handled for a specific length of time (4 min), regardless of whether a blood sample was obtained. The pilot study suggested that the capture stress paradigm is a feasible method for assessing the sensitivity of the HPA axis in kiwi.

2.2 Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to stressful stimuli is common to all avian species (Carsia and Harvey, 2000). Within seconds, catecholamines are released from the adrenal medulla, followed a few minutes later by the release of glucocorticoids from the adrenal cortex. The primary glucocorticoid in birds is corticosterone (Carsia and Harvey, 2000). To compare the sensitivity of the HPA axis of birds in different environments, and therefore their response to stressful events, Wingfield et al., (1992) developed the capture stress paradigm (CSP). The CSP involves removing blood samples at regular intervals whilst the bird is held captive, so as to describe the magnitude and duration of the corticosterone response. They do make the important assumption, however, that capture and handling is equally stressful to all individuals and species studied. The CSP does appear, though, to give a good indication of the responsiveness of the HPA axis to acute stressors in general (Wingfield et al., 1992; Wingfield et al., 1998).

The purpose of this pilot study was to test whether the CSP is a suitable method for testing the sensitivity of the HPA axis of kiwi. First, I had to determine whether kiwi respond to capture and handling with an increase in corticosterone levels. Second, I had to determine if blood samples could be collected fast enough to determine basal levels of corticosterone, as it is well known that kiwi are inherently difficult to blood sample (M. Potter pers. comm.).

2.3 Materials and Methods

All experiments were conducted with prior approval from the Massey University Animal Ethics Committee (permit no. 98/66), and the Department of Conservation. Captive nocturnal house (n=3) and outdoor pen (n=3) kiwi from Rainbow and Fairy Springs, Rotorua were used in this pilot study.

2.3.1 Blood sampling

Blood samples (0.05-0.30 ml) were taken from the right and left tarsometatarsal leg vein using a heparinised needle (25 gauge x 5/8") and 1 ml syringe. Samples were expelled into heparinised 5 ml plastic test tubes, sealed with parafilm and stored on ice for no more than 5 h before being centrifuged at 4500 rpm for 12 min (Heraeus Christ Piccolo type centrifuge). Plasma was then drawn off using a sterile needle (29 gauge) and 0.5 ml syringe, placed into a 1 ml cryotube and immediately frozen in liquid nitrogen (-187°C). The frozen plasma was later transferred to a -70°C freezer for long term storage.

2.3.2 Corticosterone response to capture and handling

2.3.2.1 Part A. Capture stress protocol

Within 5 h after dawn (outdoor pen) or 'lights on' (nocturnal house) each kiwi was removed from the nest box and blood samples taken 0, 30 and 60 minutes after the bird was picked up. During this period of one hour the kiwi remained captive in a ventilated white plastic box (39 x 29 x 30 cm), lined with newspaper and foliage. Each bird was handled until a blood sample was achieved (maximum limit was 10 min). The length of time taken to collect each sample was noted.

When kiwi were held as a pair, one individual was chosen at random and blood sampling started within 20 sec of entering the enclosure. After approximately 15 min the second bird was picked up and sampling started. A second box was used to hold this bird.

2.3.2.2 Part B. Duration of the corticosterone response to handling

After part A, each bird was returned to its nest box and any exits blocked. At either 2, 4, or 6 h later, each bird was blood sampled once more.

2.3.3 Extraction of corticosterone from plasma

All samples were included in a single extraction. Corticosterone was extracted from kiwi plasma using a method modified from Wingfield et al. (1992). Plasma samples of 20 µl were extracted into 1 ml of distilled dichloromethane. The plasma in dichloromethane was gently vortexed for 30 sec and then shaken for 1 h on a Chiltern Scientific SS70 orbital shaker. The samples were centrifuged at 3 000 rpm for 10 min at room temperature (Beckman GPR refrigerated centrifuge). An 800 µl aliquot of dichloromethane was removed from each and dried at 35°C under compressed air. Dried extracts were reconstituted in 450 µl of phosphate-buffered saline with gelatine (PBSG; 0.1M, pH 7.0), vortexed gently for 30 sec, shaken for 1 h at room temperature and then left overnight at 4°C. Samples were then frozen at -20°C until assayed. The extraction efficiency, measured using a spike of tritiated corticosterone, was 101.40 ± 0.81% ($\bar{x} \pm$ S.E.M.) (n=5).

2.3.4 Radioimmunoassay of corticosterone

All samples were assayed in duplicate within a single assay. Reconstituted extract (10 µl) was diluted with 90 µl of buffer. This was then incubated with 100 µl of antibody (Dr. R.J. Etches, University of Guelph, Ontario, Canada) and 100 µl of tritiated corticosterone (approximately 5 000 cpm; Amersham, UK) at 4°C overnight. Steroid

bound to the antibody was separated from unbound steroid by addition of 500 µl of dextran-coated charcoal (2.5 g/l charcoal, 0.25 g/l dextran in PBSG) for 15 min at 4°C. Tubes were then centrifuged at 3 000 rpm for 15 min at 4°C and the supernatant poured off. Separation by addition of dextran-coated charcoal was performed for each duplicate in succession. A 3 ml aliquot of scintillant (5 g/l PPO, 0.3 g/l dimethyl POPOP in toluene) was then added to the supernatant. The supernatant/scintillant mix was shaken for 1 h, and then left for 1 h at room temperature. Each sample was then placed on a liquid scintillation counter (Wallac 1409-411), and counted for 5 min.

Serially diluted extracted kiwi plasma was shown to be parallel to the corticosterone standard curve (Figure 2.3.1, p 17. n=3). The limit of sensitivity (smallest amount of steroid on the standard curve distinguishable from the method blank expressed as corticosterone in plasma) for kiwi plasma was 5 ng/ml. Intra-assay variation was determined previously by (Boyd, 2000). High, medium and low ($\bar{x} \pm$ S.E.M.= 1414.56 ± 54.25, 309.55 ± 13.18, 60.49 ± 9.96 pg/ml respectively) concentrations of corticosterone in PBSG were used as quality controls in each assay. These represent approximately 20, 50 and 80% binding on the standard curve. Ten duplicates of each control were used to determine intra-assay variation. High, medium and low intra-assay coefficients of variation were 13.2%, 14.8% and 13.4% respectively.

The antibody used in this assay has been shown to cross-react with various other steroids (Etches, 1976). These are deoxycorticosterone (27.9%), cortisol (6.9%), progesterone (37.6%), 11β-hydroxyprogesterone (21.3%) and <5% for oestradiol, testosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, aldosterone and pregnenolone. However, 11β-hydroxyprogesterone is an unstable intermediate in avian steroid biosynthesis, and progesterone is poorly extracted in dichloromethane (Wingfield et al., 1992). Furthermore, cortisol exists only in very low concentration in avian plasma and deoxycorticosterone has not been reported (Etches, 1976). Therefore, corticosterone is the primary steroid measured in this assay.

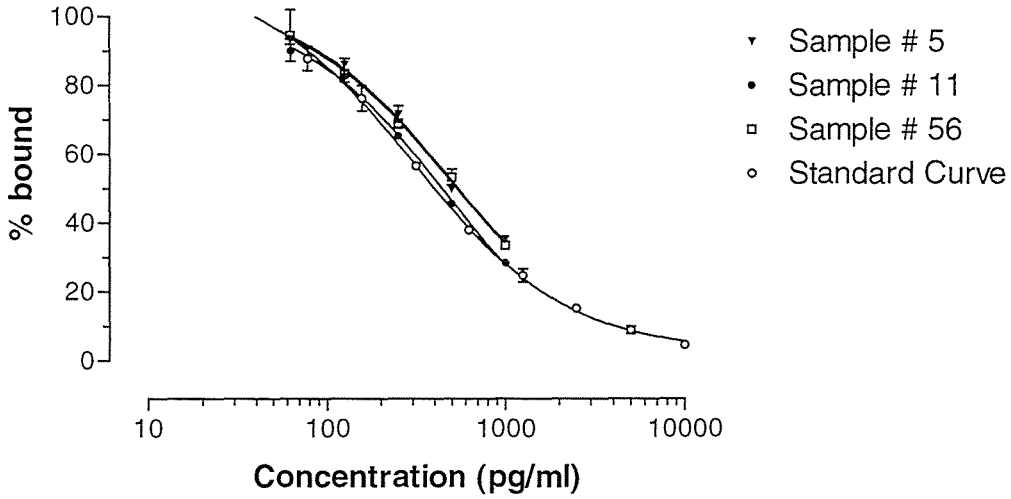


Figure 2.3.1 Parallelism demonstrated for 3 kiwi plasma samples to validate radioimmunoassay. Plasma samples were serially diluted with PBSG buffer and plotted against a PBSG buffer standard curve.

2.3.5 Statistical Analysis

Homogeneity of variance between groups was examined using a Bartlett's test (SYSTAT version 5.02, Systat Inc, Illinios). Where appropriate a two-way repeat measure ANOVA with post hoc linear contrasts were conducted using SYSTAT version 8.0 (SPSS, Inc). Linear regression analysis was performed using GraphPad Prism version 3.00 (1999; GraphPad Software Inc.).

2.4 Results

2.4.1 Corticosterone response

All kiwi from the nocturnal house (n=3) and outdoor pen (n=3) responded to capture and handling with an increase in plasma levels of corticosterone, but there was considerable variation between individuals (Figures 2.4.1, 2.4.2, pp 20, 21). Group (nocturnal house, outdoor pen) had no effect on the corticosterone response (Table 2.4.1, p 19. Figure 2.4.3, p 21). Furthermore, plasma levels of corticosterone did not differ over time for either group (Table 2.4.1, p 19. Figure 2.4.3, p 21). There was also no interaction between time and group (Table 2.4.1, p 19. Figure 2.4.3, p 21). Due to small sample sizes, statistical analysis was not possible for 60 min samples.

2.4.2 Duration of the corticosterone response

In nocturnal housed and outdoor penned kiwi, plasma levels of corticosterone had declined after 180 min. Corticosterone levels remained lower than peak levels 300 and 420 min after kiwi were first caught (Figure 2.4.4, p 22). However, due to small sample size statistical analyses at these times were not possible.

2.4.3 Gender and the corticosterone response

Gender had no effect on the corticosterone response (Table 2.4.2, p 20. Figure 2.4.5, p 22). There was also no interaction between time and gender (Table 2.4.2, p 20. Figure 2.4.5, p 22).

2.4.4 Time taken to collect the first blood sample

The plasma concentration of corticosterone immediately after capture was not related to the time taken to collect the blood sample (linear regression analysis, r^2=0.234,

P=0.331, Figure 2.4.6, p 23). The time taken to collect the blood sample also varied considerably between individuals (Figure 2.4.6, p 23).

Table 2.4.1 ANOVA table for comparison of plasma corticosterone concentrations between groups and at each sampling time.

Effect	**F**	**df**	***P***
Group	0.512	1,4	0.514
Time	7.092	1,4	0.056
Interaction of group and time	0.273	1,4	0.629
Comparison of groups for each time			
Nocturnal house vs Outdoor pen			
0 min	0.062	1,4	0.816
30 min	0.456	1,4	0.536
Comparison of times for each group			
0 min vs 30 min			
Nocturnal house	5.073	1,4	0.087
Outdoor pen	2.291	1,4	0.205

Note: The first three rows show the results of the two-way repeat measures ANOVA for the main effects and their interaction. The remaining rows show the results of post hoc comparisons of groups at each time, and comparisons of times within each group. Limited sample sizes precluded statistical analysis of 60 min samples.

Table 2.4.2 ANOVA table for comparison of plasma corticosterone concentrations between sexes and at each sampling time.

Effect	F	df	*P*
Sex	0.003	1,4	0.962
Time	6.871	1,4	0.059
Interaction of sex and time	0.140	1,4	0.727
Comparison of groups for each time			
Nocturnal house vs Outdoor pen			
0 min	0.169	1,4	0.702
30 min	0.051	1,4	0.833
Comparison of times for each group			
0 min vs 30 min			
Nocturnal house	4.486	1,4	0.102
Outdoor pen	2.525	1,4	0.187

Note: The first three rows show the results of the two-way repeat measures ANOVA for the main effects and their interaction. The remaining rows show the results of post hoc comparisons of sexes at each time, and comparisons of times within each sex. Limited sample sizes precluded statistical analysis of 60 min samples.

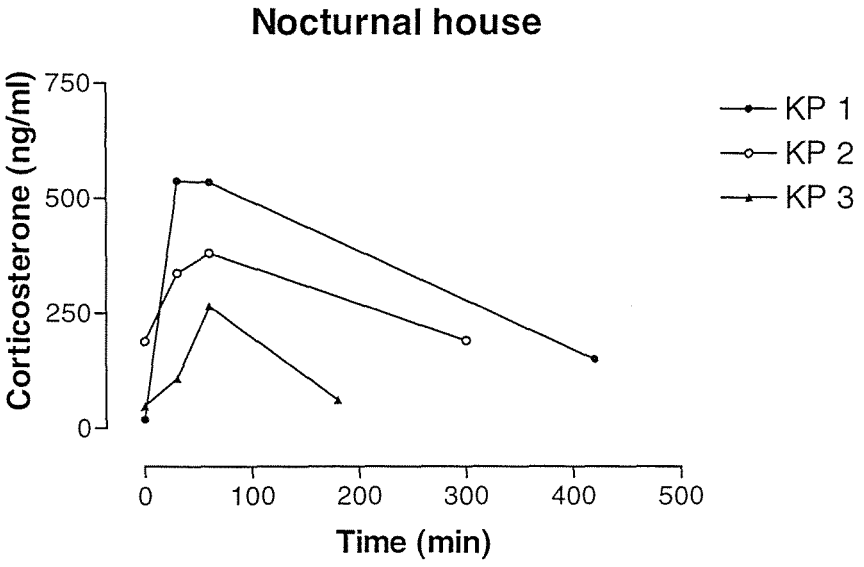


Figure 2.4.1 Individual corticosterone response curves for kiwi held in the nocturnal house.

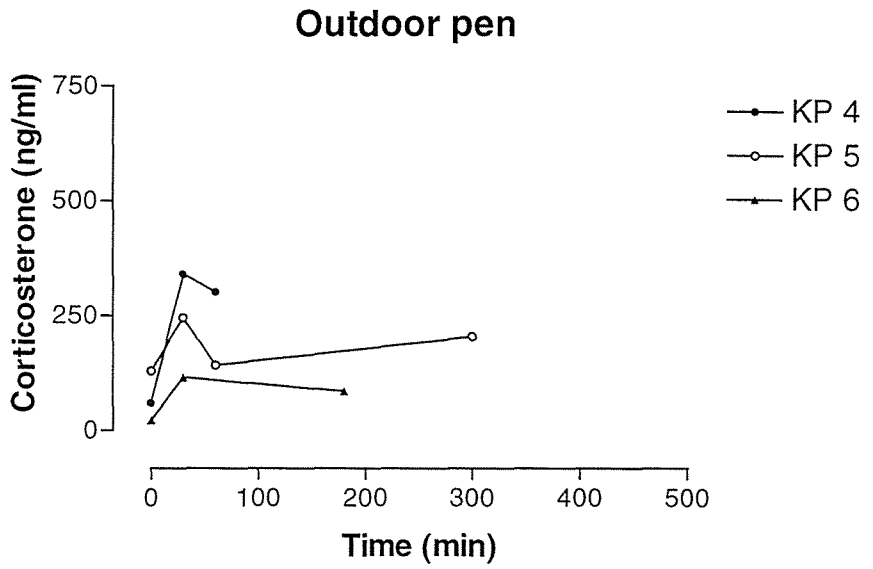


Figure 2.4.2 Individual corticosterone response curves for kiwi held in the outdoor pen.

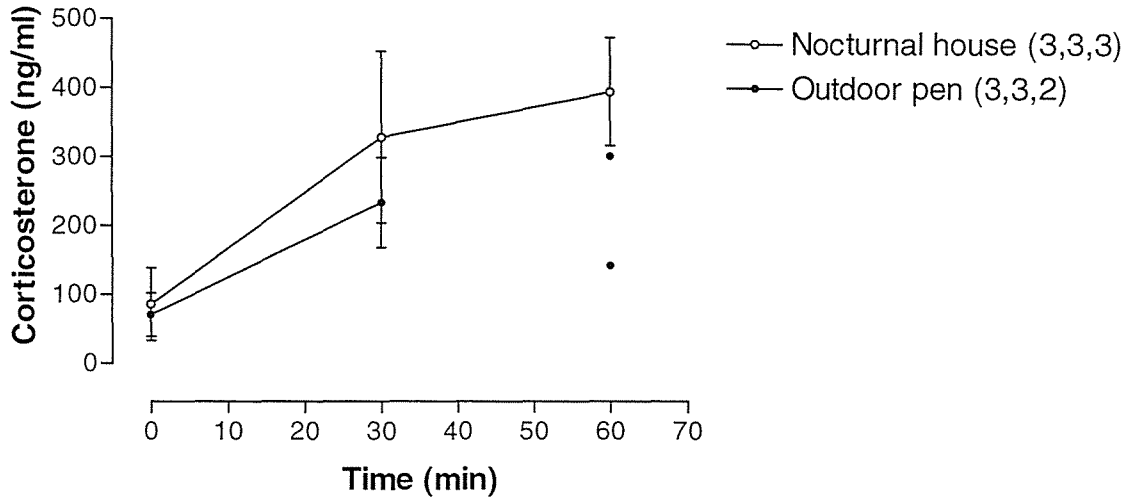


Figure 2.4.3 Changes in plasma levels of corticosterone (mean ± S.E.M.) following capture and handling in captive kiwi. Sample sizes indicated in legend.

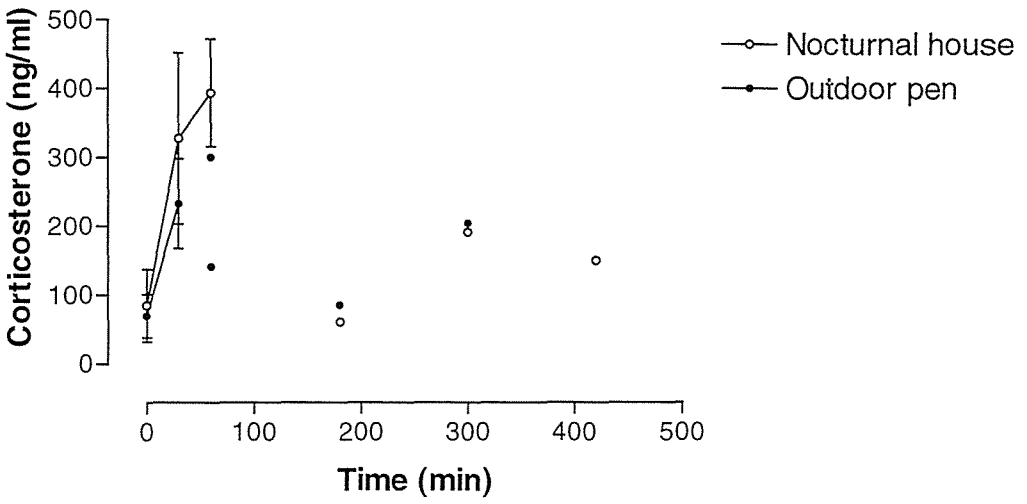


Figure 2.4.4 Plasma levels of corticosterone (mean ± S.E.M.) following capture (0-60 min), and after they were returned to their burrows (180-420 min).

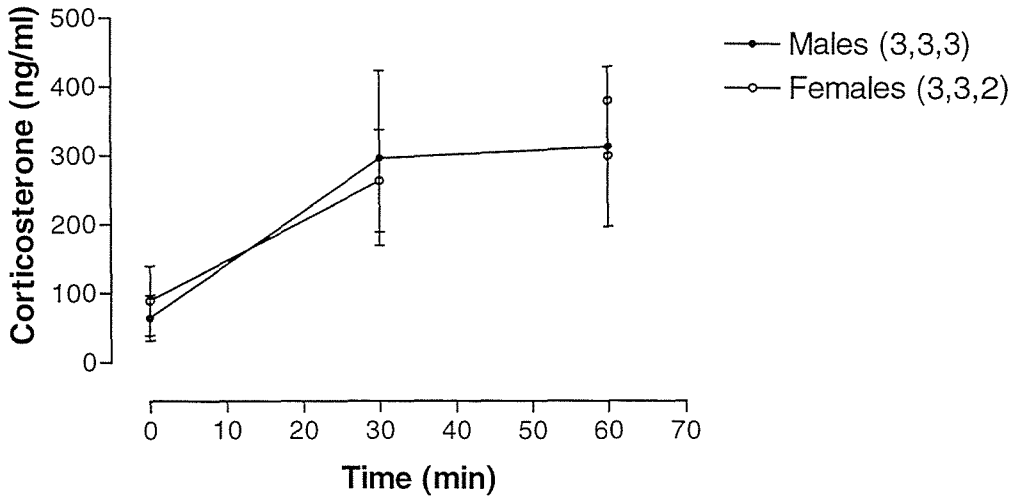


Figure 2.4.5 Changes in plasma levels of corticosterone (mean ± S.E.M.) following capture and handling, in relation to gender. Sample sizes are indicated in the legend.

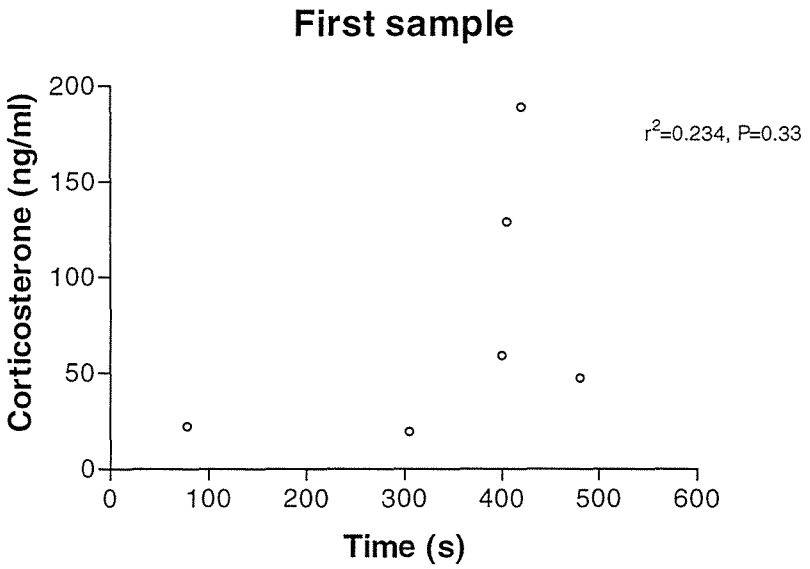


Figure 2.4.6 Relationship between the plasma concentration of corticosterone immediately after capture and the time it took to collect the blood sample.

2.5 Discussion

In nocturnal house and outdoor pen kiwi, there was almost a significant increase in corticosterone levels 30 min after capture. This lack of significance is most likely due to the considerable variation in the magnitude of the corticosterone response between individuals and small sample size. Individual variation in the corticosterone response is, however, not uncommon in birds (e.g. see Wingfield et al., 1994; Wingfield et al., 1995; Wingfield et al., 1997; Silverin, 1998; Fowler, 1999). All kiwi did however, respond to capture and handling with an increase in plasma levels of corticosterone. Examination of individual corticosterone response curves of outdoor penned kiwi revealed that levels peaked 30 min after capture and remained elevated for 60 min. Conversely, corticosterone levels of nocturnal house kiwi continued to rise between 30 min and 60 min post-capture. Corticosterone levels had also declined 2, 4 and 6 h after each bird was returned to its burrow. Sample sizes precluded statistical analysis at these times.

These results indicate that kiwi respond to capture and handling with a rapid rise in plasma levels of corticosterone similar to that seen in other species of bird (Astheimer et al., 1994; Smith et al., 1994; Wingfield et al., 1995a). However, peak levels of corticosterone are much higher than have been previously recorded for any other bird species. In fact, circulating corticosterone levels more closely resemble those of the rat (e.g. Axelrod and Reisine, 1984; Fleshner et al., 1995) than any other species of bird. Therefore, other factors controlling corticosterone secretion, metabolic clearance and influence on target tissues should be investigated. Corticosterone binding globulin greatly influences all three of these factors, and therefore should be measured.

Although gender to influences the adrenocortical response in some species (e.g. Wingfield et al., 1992; Schwabl, 1995; Hood et al., 1998), in others it does not (e.g. Smith et al., 1994; Marra et al., 1995; Dufty and Belthoff, 1997; Fowler, 1999). In kiwi, it appears also that gender has no influence on the corticosterone response.

Plasma concentrations of corticosterone are known to increase rapidly in response to stressful stimuli, such as capture and handling (Le Maho et al., 1992). For example, increases in plasma concentrations of corticosterone have been detected within seconds in chickens (*Gallus domesticus*) (Beuving and Vonder, 1978) and starlings (*Sturnus vulgaris*) (Dawson and Howe, 1983), and within a few minutes in white-crowned sparrows (*Zonotrichia leucophrys gambelli*) (Wingfield et al., 1982). Thus, the speed with which the initial sample is taken will determine whether an accurate measurement of basal corticosterone levels is obtained. In addition, the length of time a bird is initially handled can significantly affect the magnitude of the corticosterone response. For example, blackbirds (*Turdus merula*) handled for 2 or 4 min immediately after capture had a significantly lower corticosterone response than those handled for 6 or 8 min (M. Welch, unpublished data). Consequently, the time taken to collect the initial blood sample will determine whether an accurate measurement of the magnitude of the corticosterone response is achieved.

In this pilot study, there was no relationship between the time taken to collect the first blood sample and plasma corticosterone concentration. However, this may have been an artefact of small sample size, as it is well established that plasma corticosterone levels in birds generally increase within a few minutes of capture (see above for examples). It is interesting though, that plasma corticosterone levels in two samples, collected after approximately seven minutes, were a lot higher than all other initial samples. Variation in the length of time each bird was initially handled is likely to influence the magnitude and duration of the corticosterone response. Therefore, for all further sampling for this study each bird would be handled for a specific length of time (4 min), regardless of whether a blood sample was obtained. This would allow an accurate measurement of basal levels of corticosterone and yet provide enough time to collect each sample. In addition, it would provide a consistency in handling procedures between individuals.

3 Corticosterone responses to capture and handling of captive and wild northern brown kiwi

3.1 Abstract

We used a standardised stressor (capture and handling) to compare the adrenocortical response to stress between captive and wild northern brown kiwi (*Apteryx mantelli*). Repeated blood sampling was used to describe the magnitude and duration of the corticosterone response. All kiwi responded to capture and handling with a rise in plasma corticosterone levels, which peaked at 30 min (73.41 to 660.92 ng/ml). Corticosterone levels immediately after capture in wild kiwi (231.54 ± 55.79 ng/ml) were significantly higher than those of captive kiwi (nocturnal house 17.29 ± 5.85 ng/ml, outdoor pen 18.98 ± 3.98 ng/ml). This was most likely caused by the inherent delay in locating and capturing wild kiwi using radio telemetry. The corticosterone response to capture and handling in nocturnal house kiwi (200.06 ± 34.82 ng/ml) was significantly lower than in wild kiwi (415.22 ± 42.79 ng/ml), but similar to those in outdoor penned kiwi (330.80 ± 55.64 ng/ml). The cause of these differences is unclear. Nocturnal house kiwi may have become habituated to the presence of humans. Alternatively, the difference may be due to nocturnal house kiwi being held on a different light cycle to outdoor pen and wild kiwi. Corticosterone levels in nocturnal house kiwi returned to basal (43.77 ± 23.29 ng/ml) 2 h after capture and handling. Regularly handled kiwi appear not to respond to public display and handling with an increase in plasma levels of corticosterone. Low basal levels of corticosterone indicate that captive kiwi have acclimatised to captivity. Furthermore, the adrenocortical response to capture and handling of captive kiwi was equivalent to or lower than that of wild kiwi. Therefore, this study suggests that captive kiwi do not respond to capture and handling with a chronic elevation in plasma levels of corticosterone.

3.2 Introduction

Birds respond to stressful stimuli with an activation of the hypothalamic-pituitary-adrenal (HPA) axis (Siegel, 1980; Carsia and Harvey, 2000), with corticosterone being the principal adrenocortical hormone released when the HPA axis is stimulated (Carsia and Harvey, 2000). Acute rises in corticosterone can favour immediate survival and long term reproductive success (reviewed in Silverin, 1998b; Wingfield et al., 1998). However, extended periods of stressful conditions can result in chronic elevations of corticosterone (Silverin, 1998b). This in turn has been shown to suppress the immune and reproductive systems, promote severe protein loss and inhibit growth (reviewed in Wingfield et al., 1998).

The development of a standardised handling protocol by Wingfield et al. (1992) has enabled researchers to compare the sensitivity of the HPA axis of birds in different circumstances. For example, the sensitivity of the HPA axis to stressors has been shown to vary between different life stages (e.g. breeding, moult and migration, see Wingfield et al., 1998 for review) and habitats (Marra and Holberton, 1998). Regular association with, or handling by humans can also cause a decline in corticosterone secretion (Barnett et al., 1994; Fowler, 1999; Collette et al., 2000). Furthermore, captivity *per se* may also influence corticosterone secretion. For example, short periods of captivity cause an increase in corticosterone secretion (Marra et al., 1995; Romero and Wingfield, 1999). However after longer periods, birds appear to acclimatise to captivity, with corticosterone levels starting to approximate those of wild conspecifics (Dufty and Belthoff, 1997; Kitaysky et al., 1999a). Captivity has also been shown to extinguish annual rhythms in the corticosterone response of white-crowned sparrows (*Zonotrichia leucophrys gambelli*) (Breuner et al., 1999; Romero and Wingfield, 1999). Consequently, comparisons of the adrenocortical stress response in this manner has allowed researchers to identify chronically stressed populations (i.e. chronic elevations of corticosterone), and those populations with increased susceptibility to stress (i.e. increased sensitivity of the HPA axis, Wingfield et al., 1997).

In New Zealand, kiwi (*Apteryx mantelli*) are held in captivity for display and public education and for captive breeding (refer to text box on p 10). For the former, kiwi are

held in nocturnal houses where the light cycle is reversed so that the public can view the birds through glass-fronted enclosures during the day. These enclosures allow a limited amount of aural and visual interaction with the public. A second type of nocturnal house enclosure at Willowbank, Christchurch, allows considerably more interaction between the public and the kiwi. There, only a low wooden fence, rather than a glass wall, separates the public from the kiwi. For captive breeding, kiwi are held in outdoor pens. Most of these are secluded, positioned well away from the public to avoid any disturbance. However, a few breeding facilities allow public access close to outdoor enclosures. There are also a small number of kiwi that are brought out of their enclosures and handled regularly for display to the public. A typical display session would involve the removal of the bird from its enclosure and placing it on the ground (or in an arena) within very close proximity to unfamiliar humans for periods up to half an hour. The effects of these various management systems on the stress response of captive kiwi are unknown.

Conservation strategies should work to minimise the occurrence of stressful situations, which are likely to result in chronically elevated levels of corticosterone. Consequently, identification of such situations would yield important information for conservation management. The objective of this study was to compare the basal levels of corticosterone and the corticosterone response of kiwi in different management systems (including the wild), by using a standardised handling protocol. This would help identify captive management systems that kiwi deem to be stressful (i.e. increased sensitivity of the HPA axis).

3.3 Materials and methods

All experiments were conducted with prior approval from the Massey University Animal Ethics Committee (permit no. 98/66), and the Department of Conservation.

3.3.1 Sourcing kiwi

3.3.1.1 Captive kiwi

Captive kiwi were held in nocturnal houses and outdoor pens at institutes throughout New Zealand. The characteristics of each enclosure varied between institutes (see tables 3.3.1, p 33 and 3.3.2, p 34 for summary). Of the regularly handled kiwi, two were held in nocturnal houses, and one was held in an outdoor pen.

3.3.1.2 Wild kiwi

Blood samples were collected from wild kiwi that had previously been radio-tagged (two-stage radio transmitter; Sirtrack, New Zealand) at Aotuhia, Taranaki (39°14'S, 174°48'E). Kiwi were located using a TR4 receiver (Telonics, USA) and a three element yagi aerial. These kiwi are handled only every 6-12 months when the radio-tag is changed. The most recent replacement of a radio-tag was 6 months prior to our study (P. Horton pers. comm.). Therefore, these birds would be relatively naive to capture and handling.

3.3.2 Blood sampling

All blood samples were collected using the method outlined in section 2.3.1.

3.3.3 Experiment 1. Corticosterone response to capture and handling

3.3.3.1 Part A. Capture stress protocol

Within 5 h after dawn (outdoor pen and wild) or 'lights on' (nocturnal house) each kiwi was removed from the nest box (captive) or burrow (wild) and blood samples taken 0, 30 and 60 minutes after the bird was picked up. During this period of one hour the kiwi remained captive in a ventilated white plastic box (39 x 29 x 30 cm) lined with newspaper and foliage. They were handled for 4 min at each sample time and then returned to the box, regardless of whether a blood sample was obtained. The time taken to collect the sample (within the 4 min handling period) was noted.

When kiwi were held as a pair in captivity, one individual was chosen at random and blood sampling started within 20 sec of entering the enclosure. After approximately 15 min the second bird was picked up and sampling started. A second box was used to hold this bird. When a pair of kiwi were found together in the wild, one individual was removed at random, and blood sampling started. Once the first kiwi had been blood sampled (at 0, 30 and 60 min), the second kiwi was removed from the burrow and sampling started. However, due to the difficulty and noise associated with locating kiwi using radio telemetry, it is possible that wild kiwi were aware of our presence some 5-18 min prior to capture. The corticosterone responses of kiwi sampled second were analysed separately to those sampled first.

3.3.3.2 Part B. Duration of the corticosterone response to handling

After part A, each bird was returned to its nest box or burrow (where possible) and any exits blocked. At either 2, 4, 6 or 8 h later, each bird was sampled once more. In the wild, it was not always practical for the bird to be returned to the burrow. In such cases they were kept in the box and sampled at either 1.5 or 2 h after the end of part A.

3.3.4 Experiment 2. Effect of handling time on wild kiwi

Within 5 h after dawn, kiwi were removed from their burrows in the wild and blood samples taken at 0, 30 and 60 min after the bird was picked up. Between samples, the birds were held in a ventilated white plastic box. Treatment birds were handled initially for 15 min (blood sample obtained within 4 min), and then for only 4 min at each of the two subsequent sampling times (30 and 60 min). Control birds were handled for 4 min at every sample time (0, 30 and 60 min).

3.3.5 Experiment 3. Effect of regular handling on the corticosterone response

Individual kiwi accustomed to regular handling were picked up by their regular handlers and placed down in close proximity to at least two strangers, mimicking the way they are usually handled for public display. After 30 min on display, a single blood sample was taken. Seven days later, the bird was blood sampled immediately after removal from its nest box.

3.3.6 Morphometric data

Once the final blood sample had been taken, each bird was weighed using a 10 kg spring balance (± 50 g). Toe lengths were measured using vernier callipers.

3.3.7 Extraction of corticosterone from plasma

The extraction of corticosterone from the plasma of kiwi was conducted using the methodology outline in section 2.3.3, with the following modifications. All samples from an individual bird were extracted within the same extraction, with birds being randomly allocated to four different groups for extraction. Samples of tritiated quail plasma were included in each extraction to check that the extraction efficiency remained consistent between extractions (table 3.3.3, p 35).

3.3.8 Radioimmunoassay of corticosterone

The radioimmunoassay of corticosterone in kiwi plasma was conducted using the methodology outlined in section 2.3.4, with the following modifications. Birds (and therefore samples) were assayed in the same groups (four) as they were extracted in. Duplicates of the quality controls were included at the beginning and end of each assay to calculate inter-assay variation. The inter-assay coefficients of variation for ten assays were 11.9%, 16.2% and 19.5% for high, medium and low quality controls respectively.

3.3.9 Statistical analysis

Analysis of homogeneity of variance between groups was performed using a Bartletts test (SYSTAT version 5.02, Systat Inc, Illinois). Where appropriate the following statistical analyses were conducted using SYSTAT version 8.0 (SPSS, Inc); two-way and one-way repeat measures ANOVA with post hoc linear contrasts, one-way single measures ANOVA with Bonferroni post hoc comparisons, and students t-test. Calculation of the area under the corticosterone curve (integrated corticosterone response), and linear regression were performed using GraphPad Prism version 3.00 (1999; GraphPad Software Inc.), using the trapezoid method.

Table 3.3.1 Summary of the source and number of kiwi sampled from outdoor pens, and the characteristics of the enclosures in which they were housed. N/A= not applicable.

Institute	Number of Birds Sampled		Number of birds housed as a;		Type of barrier between enclosures				Public access close to nesting box
	♂	♀	Single	Pair	Aural	Visual	Tactile	Physical	
Auckland Zoo	2	2	2	2	--	--	✔	✔	NO
Moana Reserve	1	1	--	2	N/A	N/A	N/A	N/A	YES
Orana Park	1	1	--	2	N/A	N/A	N/A	N/A	NO
Rainbow Springs	1	--	1	--	--	✔	✔	✔	NO
Westshore Wildlife Reserve	1	1	1	1	--	✔	✔	✔	NO
Whangarei Native Bird Recovery Centre	1	--	1	--	N/A	N/A	N/A	N/A	NO
Willowbank Wildlife Reserve	2	2	--	4	--	--	--	✔	YES
Whakarewarewa	1	1	2	--	✔	✔	✔	✔	NO

Table 3.3.2 Summary of the source and number of kiwi sampled from nocturnal houses, and the characteristics of the enclosures in which they were housed. N/A= not applicable.

Institute	Number of Birds Sampled		Number of birds housed as a;			Type of barrier between enclosures				Public Viewing	Light Cycle Light/Dark	Shut out of burrow while on display
	♂	♀	Single	Pair	Same Sex Pair	Aural	Visual	Tactile	Physical			
Moana Reserve	1	1	--	2	--	N/A	N/A	N/A	N/A	Glass Wall	16L:8D	YES
Napier Kiwi House	2	--	2	--	--	--	--	--	✔	Glass Wall	19L:5D	YES
National Wildlife Centre	2	--	2	--	--	--	--	--	✔	Glass Wall	13.5L:10.5D	NO
Orana Park	1	1	--	2	--	N/A	N/A	N/A	N/A	Glass Wall	16.75L:7.25D	NO
Queenstown Kiwi and Birdlife Park	1	2	--	3	--	✔	✔	✔	✔	Glass Wall	15L:9D	YES
Rainbow Springs	--	1	1	--	--	--	✔	✔	✔	Glass Wall	14L:10D	YES
Willowbank Wildlife Reserve	4	2	--	4	2	--	--	--	✔	Wooden Fence	11.75L:12.25D	YES
Whakarewarewa Thermal Reserve	--	2	--	--	2	N/A	N/A	N/A	N/A	Glass Wall	14L:10D	YES

Table 3.3.3 Extraction efficiencies of kiwi plasma, quail plasma and labelled buffer. These were used to confirm consistency in extraction efficiencies between extractions.

	Mean (%)	**S.EM. (%)**	**CV (%)**
Kiwi	101.40	0.81	1.77
Quail	103.20	0.95	2.07
20 µl Label	103.03	1.16	1.95

3.4 Results

3.4.1 Comparison of corticosterone responses between groups

All kiwi responded to capture and handling with a rise in plasma levels of corticosterone. However, there was considerable variation in the size of the response between individuals (Figures 3.4.1, 3.4.2, 3.4.3, pp 42-44). Group (nocturnal house, outdoor pen or wild) and time had significant effects on corticosterone levels (two-way repeat measures ANOVA, Figure 3.4.4, p 45 Table 3.4.1, p 46). There was also a significant interaction between these two variables.

Immediately after capture, plasma levels of corticosterone were low in nocturnal house and outdoor pen kiwi, and significantly elevated in wild kiwi (Figure 3.4.4, p 45 Table 3.4.1, p 46). Corticosterone levels continued to rise in nocturnal house and outdoor pen kiwi to peak levels 30 min after capture and handling and remained elevated for 60 min (Table 3.4.1, p 46). In contrast, there was no significant increase in plasma levels of corticosterone in wild kiwi 30 min or 60 min after capture (Table 3.4.1, p 46). However, all (except one) individual wild kiwi did show an increase in corticosterone levels 30 min after capture (Figure 3.4.3, p 45).

In nocturnal house kiwi, peak levels of corticosterone 30 min after capture were significantly lower than those of wild kiwi, and almost lower than those found in outdoor pen kiwi (Table 3.4.1, p 46). Plasma levels of corticosterone 60 min after capture in nocturnal house kiwi continued to be significantly lower than those found in wild kiwi. There was, however, no significant difference in corticosterone levels between nocturnal house and outdoor pen kiwi 60 min after capture (Table 3.4.1, p 46). There was also no significant difference in plasma levels of corticosterone between outdoor penned and wild kiwi at either 30 min or 60 min after capture.

3.4.2 Duration of the corticosterone response

In nocturnal house kiwi, corticosterone levels had returned to basal 180 min after the end of the capture stress protocol (two sample t-test, t=1.746, df=3.2, P=0.173), and remained there 300 min later (one way repeat measures ANOVA, $F_{1,2}$=0.612, P=0.516, Figure 3.4.5, p 45). Corticosterone levels had dropped significantly after 420 min in outdoor penned kiwi ($F_{1,2}$=56.656, P=0.017), but were still elevated above basal ($F_{1,2}$=48.375, P=0.020, Figure 3.4.5, p 45).

3.4.3 Corticosterone response and body mass

There was a moderate correlation between basal corticosterone levels and body mass in outdoor pen kiwi (r^2 =0.575, P=0.048, Figure 3.4.6, p 47). Basal corticosterone levels and body mass were not related for any other group, or when all groups were combined (Figure 3.4.6, p 47).

A marked inverse relationship between corticosterone levels and body mass in nocturnal house kiwi existed 30 min after capture (r^2 =0.723, P=0.008, Figure 3.4.7, p 48). However, no relationship existed between plasma corticosterone levels and body mass for any other group, or when all groups were combined (Figure 3.4.7, p 48).

There was also a marked relationship between corticosterone levels and body mass in nocturnal house kiwi after 60 min (r^2=0.854, P<0.001, Figure 3.4.8, p 49). Again, no relationship existed between plasma corticosterone levels and body mass for any other group, or when all groups were combined (Figure 3.4.8, p 49).

3.4.4 Corticosterone response and condition index

The index of condition (body mass/toe length^3) was not correlated with plasma corticosterone levels at any time for any group (Figures 3.4.9, 3.4.10, 3.4.11, pp 50-52).

3.4.5 Gender and the corticosterone response

There was no difference in basal corticosterone levels between male and female kiwi in the nocturnal houses (t=1.446, df=6, *P*=0.221), nor after 60 min (t=0.822, df=7, *P*=0.474, Figure 3.4.12, p 53). Males and females in the outdoor pens did not differ in their corticosterone responses at 0 min (t=0.956, df=6, *P*=0.377), 30 min (t=0.914, df=5, *P*=0.421) or 60 min (t=0.576, df=6, *P*=0.588, Figure 3.4.12, p 53). There was also no difference in corticosterone responses between wild male and female kiwi either at 0 min (t=0.278, df=8, *P*=0.788) or after 30 min, (t=0.839, df=8, *P*=0.448, Figure 3.4.12, p 53). Due to difficulties in gaining repeated blood samples from some individuals, analysis of these data had to be performed using t-tests, rather than a two-way repeat measures ANOVA.

3.4.6 Time since first disturbed

All blood samples were collected within 4 min of capture. There was no correlation between initial plasma levels of corticosterone and the time taken to collect the first sample within the 4 min (Figure 3.4.13, p 54). There was also no correlation between plasma corticosterone levels and the time elapsed since captive (nocturnal house, outdoor pen) kiwi were first disturbed (Figure 3.4.14, p 54). The time taken to capture wild kiwi, i.e. the time since they were first disturbed, was estimated for each bird to be between 5 and 18 min. There was no correlation between the time taken to capture wild kiwi and plasma corticosterone levels immediately after capture (Figure 3.4.15, p 55).

3.4.7 Rate of corticosterone increase

The rate of increase in plasma levels of corticosterone was not significantly different between kiwi housed in the nocturnal house and outdoor pen (two sample t-test, t=1.941, df=11, *P*=0.085, Figure 3.4.16, p 55). However, it would be incorrect to statistically compare the rate of corticosterone increase of wild kiwi to those of

nocturnal house or outdoor penned kiwi, because plasma corticosterone levels were already elevated in wild kiwi when they were captured.

3.4.8 Comparisons of body mass

Wild female kiwi were significantly heavier than wild male kiwi (two sample t-test, t=3.183, df=7, P=0.021, Figure 3.4.17, p 56). In contrast, there was no difference between the sexes in captive kiwi (nocturnal house t=0.694, df=7, P=0.510; outdoor pen t=1.746, df=10, P=0.121). A comparison of weights between groups indicated that nocturnal house females were significantly lighter than wild females (one way single measures ANOVA, $F_{2,12}$=6.274, P=0.014), and that outdoor pen males were significantly lighter than wild males ($F_{2,16}$=4.591, P=0.027). Male captive kiwi were significantly lighter than wild males (t=2.956, df=17, P=0.014) when the weights from nocturnal house and outdoor penned male kiwi were combined. Also, female captive kiwi were significantly lighter than wild females (t=5.167, df=13, P<0.001) when the weights from nocturnal house and outdoor penned female kiwi were combined.

3.4.9 The integrated corticosterone response (area under the curve)

The integrated corticosterone response was not significantly different between kiwi housed in the nocturnal house and outdoor pen (two sample t-test, t=2.063, df=11, P=0.074, Figure 3.4.18, p 56). Plasma corticosterone levels immediately after capture in wild kiwi were elevated, most likely because of our sampling methods. It is incorrect to statistically compare the integrated corticosterone response of wild kiwi to those of nocturnal house and outdoor penned kiwi, because plasma corticosterone levels were already elevated in wild kiwi when they were captured.

There was a marked relationship between the integrated corticosterone response and body mass in nocturnal house kiwi (linear regression, r^2=0.7817, P=0.0082, Figure 3.4.19, p 57). No such relationship existed for any other group (Figure 3.4.19, p 57).

3.4.10 The effect of handling duration on the corticosterone response of wild kiwi

The duration of handling (4 min vs 15 min) immediately after capture had no effect on the corticosterone response of wild kiwi (two-way repeat measures ANOVA, Table 3.4.2, p 59. Figure 3.4.20, p 58). There was a significant effect of time on the corticosterone response. However, there was no interaction between handling duration and time.

Corticosterone levels rose significantly after 30 min in kiwi handled initially for 4 min, and almost significantly in kiwi that were handled initially for 15 min (Table 3.4.2, p 59). There was no significant change in corticosterone levels between 30 min and 60 min after capture in either group.

3.4.11 Comparison of captive management systems

There was no difference in basal levels of corticosterone between kiwi housed in the nocturnal house and those at Willowbank (a walk through enclosure) (t=0.255, df=9.0, P=0.804). There was, however, a tendency for levels at 30 min and 60 min to be higher at Willowbank (Figure 3.4.21, p 60).

Corticosterone levels tended to be similar between kiwi held in outdoor pens and those held in public access pens at 0 min and 30 min after capture (Figure 3.4.22, p 60). There was no significant difference between the two groups after 60 min (t=0.116, df=2.9, P=0.915).

3.4.12 Corticosterone responses of birds housed singly or in pairs

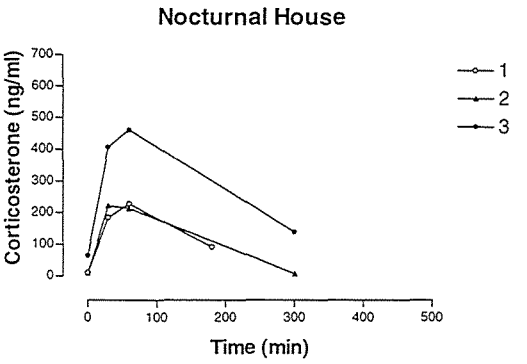
There was no difference in basal levels of corticosterone between kiwi housed singly or as a pair in nocturnal houses (two sample t-test, t=1.446, df=4, P=0.221, Figure 3.4.23, p 61). There was also no difference in corticosterone levels at 30 min (t=0.419, df=6, P=0.698) or 60 min (t=0.636, df=7, P=0.549) between kiwi housed singly or as a pair in the nocturnal house. In addition, plasma levels of corticosterone 60 min after capture

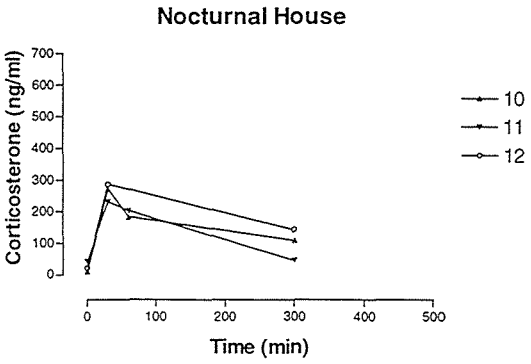
did not differ between kiwi housed singly or as a pair in the outdoor pens (t=0.044, df=6, *P*=0.967, Figure 3.4.23, p 61). Initial levels of corticosterone in wild kiwi did not differ between kiwi found by themselves or with their mate (two sample t-test, t=2.144, df=8, *P*=0.067, Figure 3.4.23, p 61). Due to difficulties in gaining repeated blood samples from some individuals, analysis of these data had to be performed using t-tests, rather than a two-way repeat measures ANOVA.

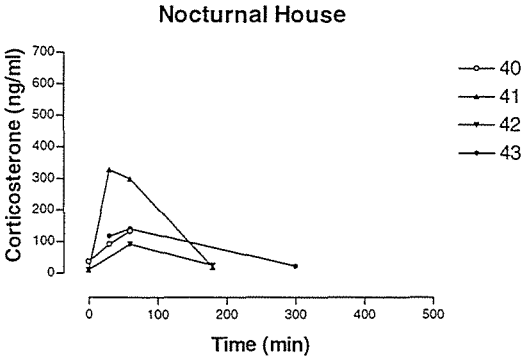
When wild kiwi were found as a pair, each bird was sampled in succession (see methods section 3.3.3.1 Part A). Kiwi removed from the burrow first had significantly higher initial levels of corticosterone than kiwi removed second (two sample t-test, t=2.580, df=11, *P*=0.028, Figure 3.4.24, p 62). Plasma levels of corticosterone after 30 min continued to be higher in kiwi sampled first (t=3.377, df=11, *P*=0.006). In nocturnal house kiwi, corticosterone levels at 30 min were lower in kiwi sampled first compared to those sampled second (t=2.649, df=9, *P*=0.048, Figure 3.4.24, p 62). In addition, plasma levels of corticosterone 60 min after capture tended to be higher in nocturnal house kiwi sampled second compared to those sampled first (Figure 3.4.24, p 62). Statistical comparisons between outdoor penned birds removed first and second were not possible, due to small sample size.

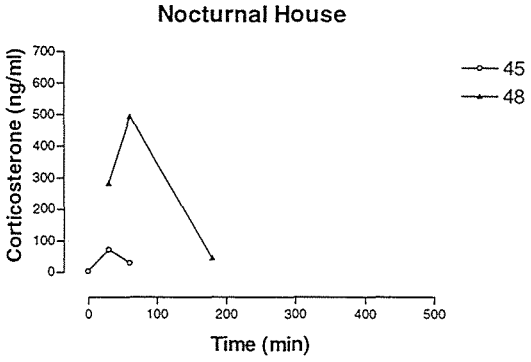
3.4.13 The corticosterone response of regularly handled kiwi

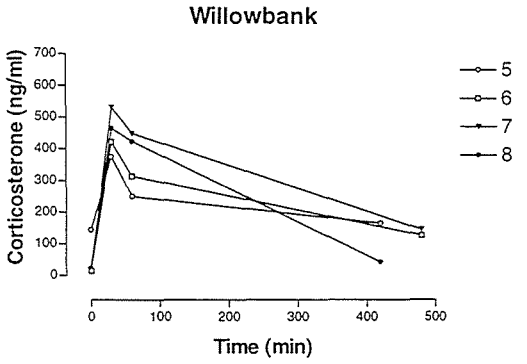
A comparison of individual values showed that corticosterone values were lower after 30 min of handling and public display than immediately prior to display (Figure 3.4.25, p 63). Due to difficulties in sample collection, small sample size prevented statistical analysis of corticosterone levels before and after a period of public display.











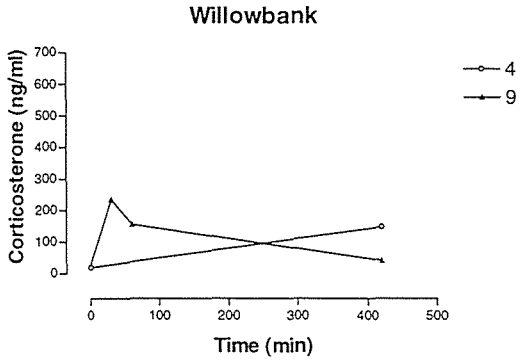
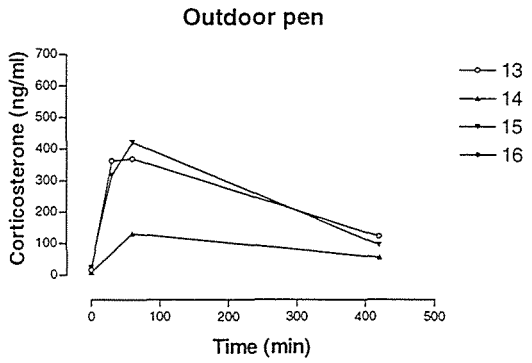
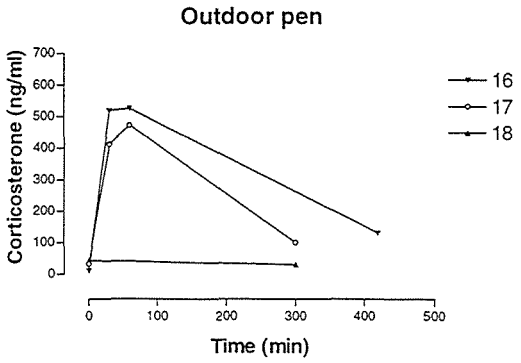
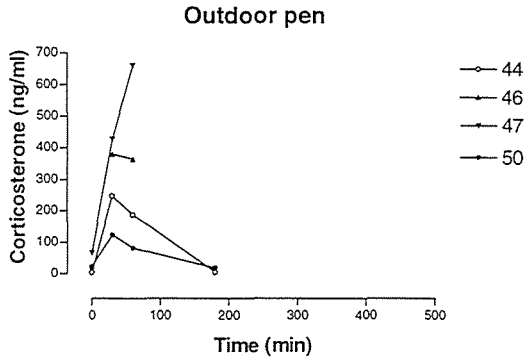
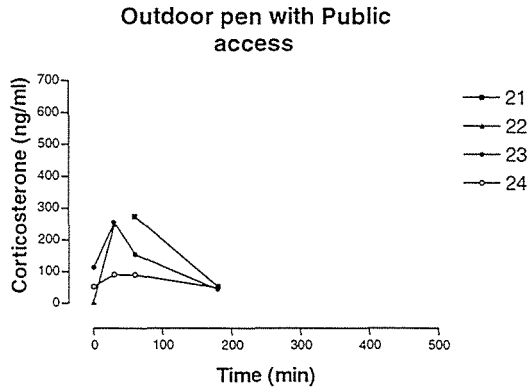


Figure 3.4.1 Individual corticosterone response curves for kiwi held in nocturnal houses and at Willowbank (a walk through enclosure). Numbers in the legends refer to individual kiwi numbers.









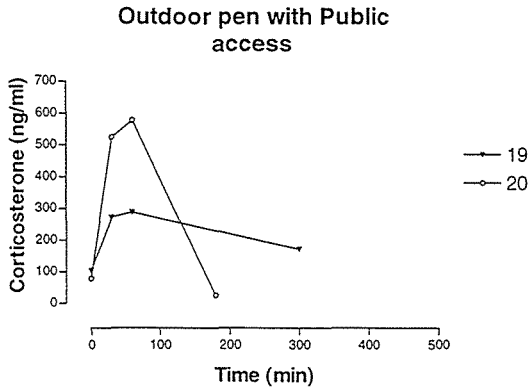
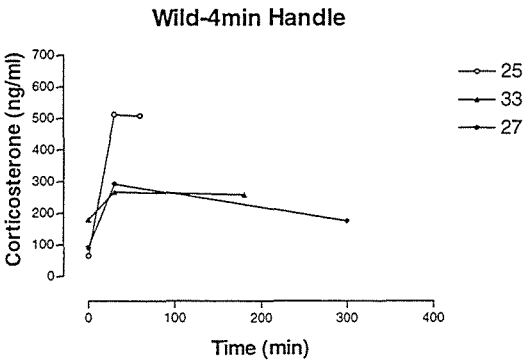
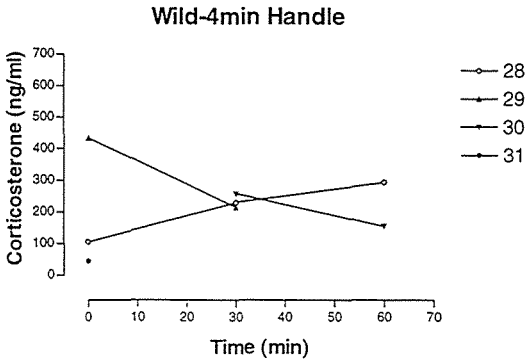
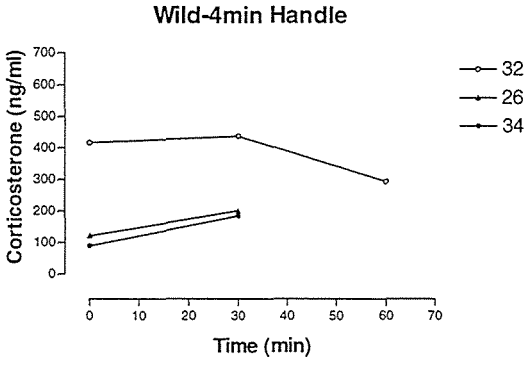
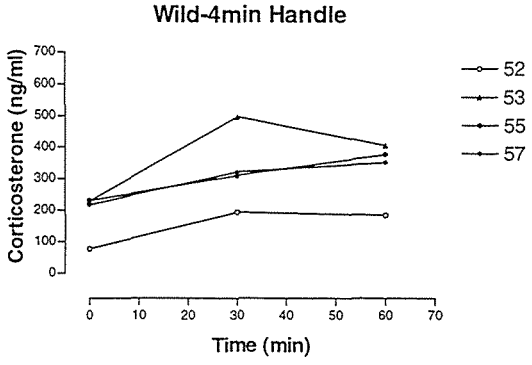


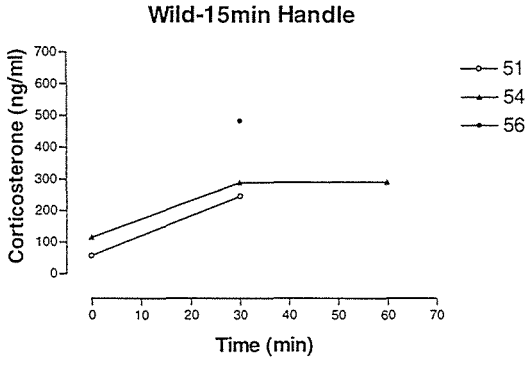
Figure 3.4.2 Individual corticosterone response curves of kiwi held in outdoor pens, and pens with close public access. Numbers in the legends refer to individual kiwi numbers.











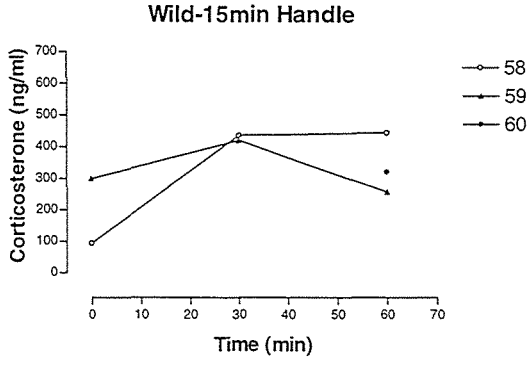


Figure 3.4.3 Individual corticosterone response curves for kiwi found in the wild and handled initially for 4 min or for 15 min. Numbers in the legends refer to individual kiwi numbers.

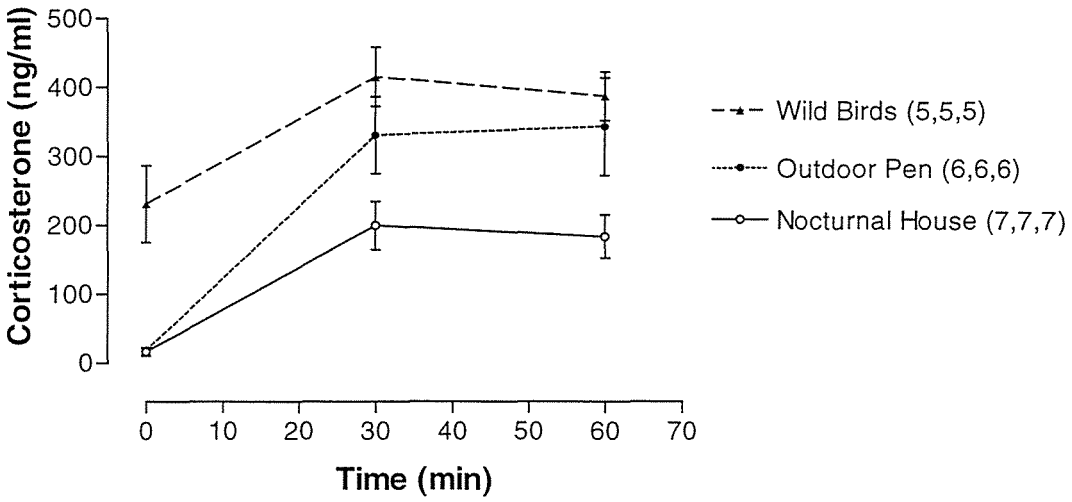


Figure 3.4.4 Changes in plasma levels of corticosterone (mean ± S.E.M.) following capture and handling in kiwi. Sample sizes are indicated in the legend.

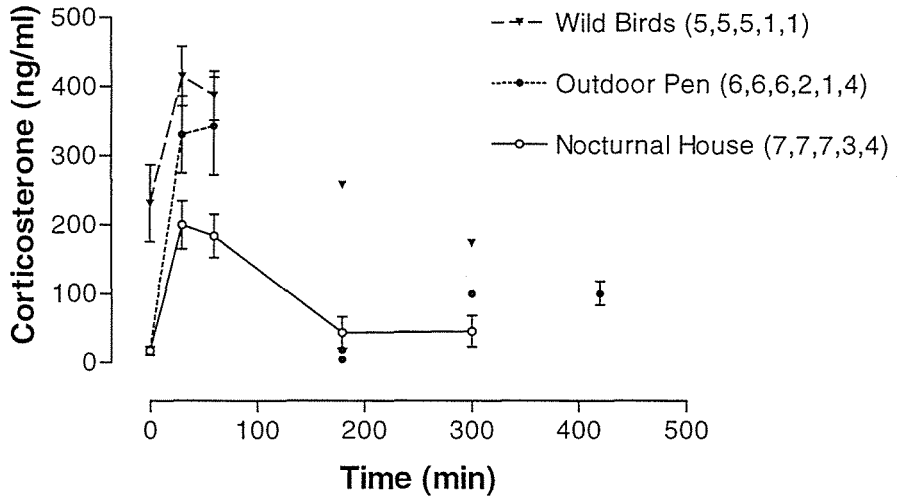


Figure 3.4.5 Plasma levels of corticosterone (mean ± S.E.M.) following capture (0-60 min), and after they were returned to their burrows (180-420 min). Sample sizes are indicated in the legend.

Table 3.4.1 ANOVA table for comparison of plasma corticosterone concentrations between groups and at each time.

Effect	F	df	*P*
Group	16.407	2,15	<0.001
Time	87.682	2,30	<0.001
Interaction of group and time	9.073	4,30	<0.001
Comparison of groups for each time			
Nocturnal house vs Outdoor pen			
0 min	0.361	1,15	0.557
30 min	4.032	1,15	0.063
60 min	3.071	1,15	0.100
Nocturnal house vs Wild			
0 min	41.164	1,15	<0.001
30 min	8.820	1,15	0.010
60 min	5.745	1,15	0.030
Outdoor pen vs Wild			
0 min	31.943	1,15	<0.001
30 min	1.054	1,15	0.321
60 min	0.501	1,15	0.490
Comparison of times for each group			
0 min vs 30 min			
Nocturnal house	61.815	1,15	<0.001
Outdoor pen	65.111	1,15	<0.001
Wild	3.249	1,15	0.092
0 min vs 60 min			
Nocturnal house	54.295	1,15	<0.001
Outdoor pen	61.577	1,15	<0.001
Wild	2.622	1,15	0.126
30 min vs 60 min			
Nocturnal house	1.186	1,15	0.293
Outdoor pen	0.101	1,15	0.755
Wild	0.200	1,15	0.661

Note: The first three rows show the results of the two-way repeat measures ANOVA for the main effects and their interaction. The remaining rows show the results of post hoc comparisons of groups at each time, and comparisons of times within each group.

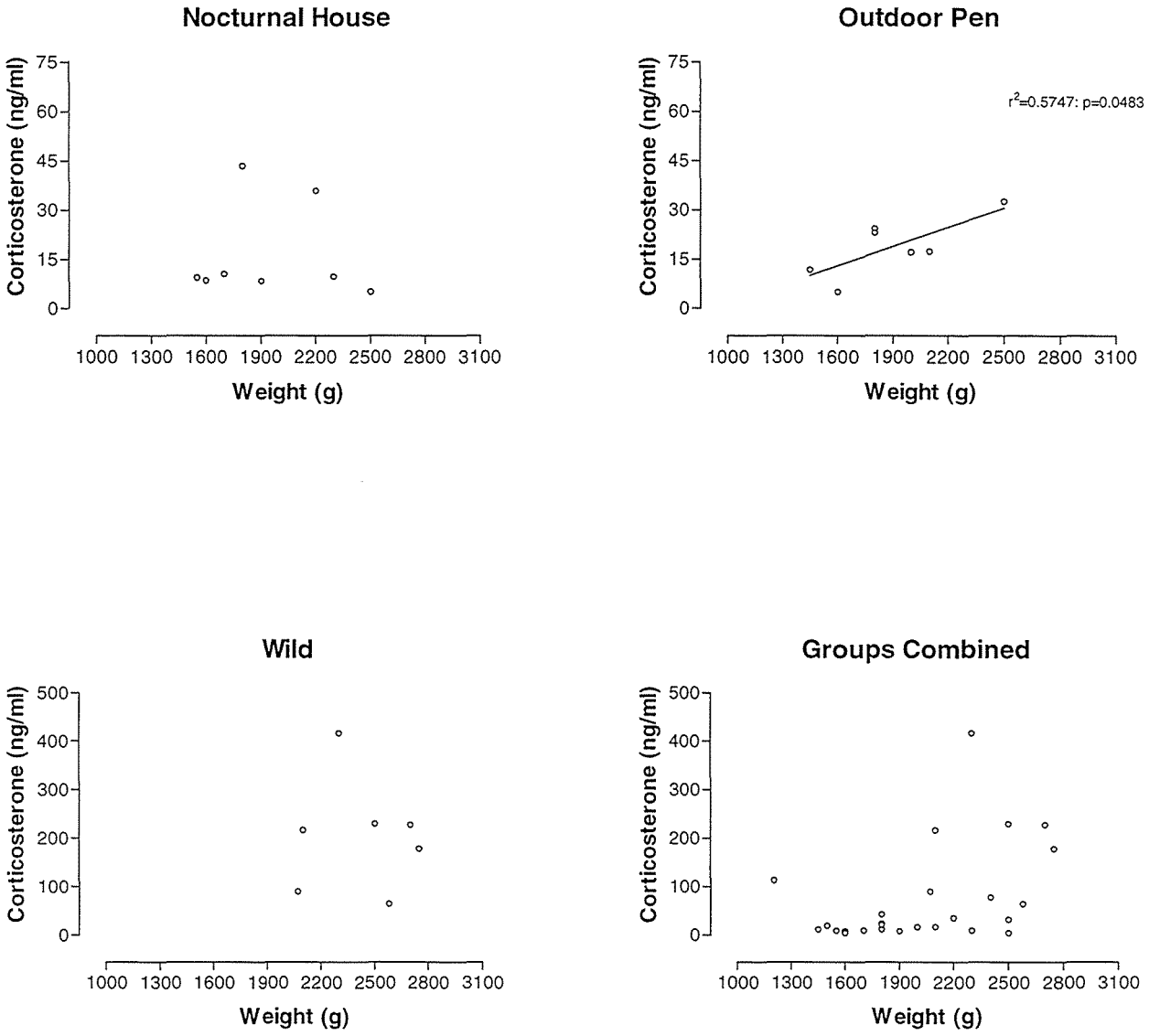


Figure 3.4.6 Correlations between body mass and corticosterone levels immediately after capture. Significant correlations are indicated on the graph. Note the difference in y-axis scale in the lower graphs.

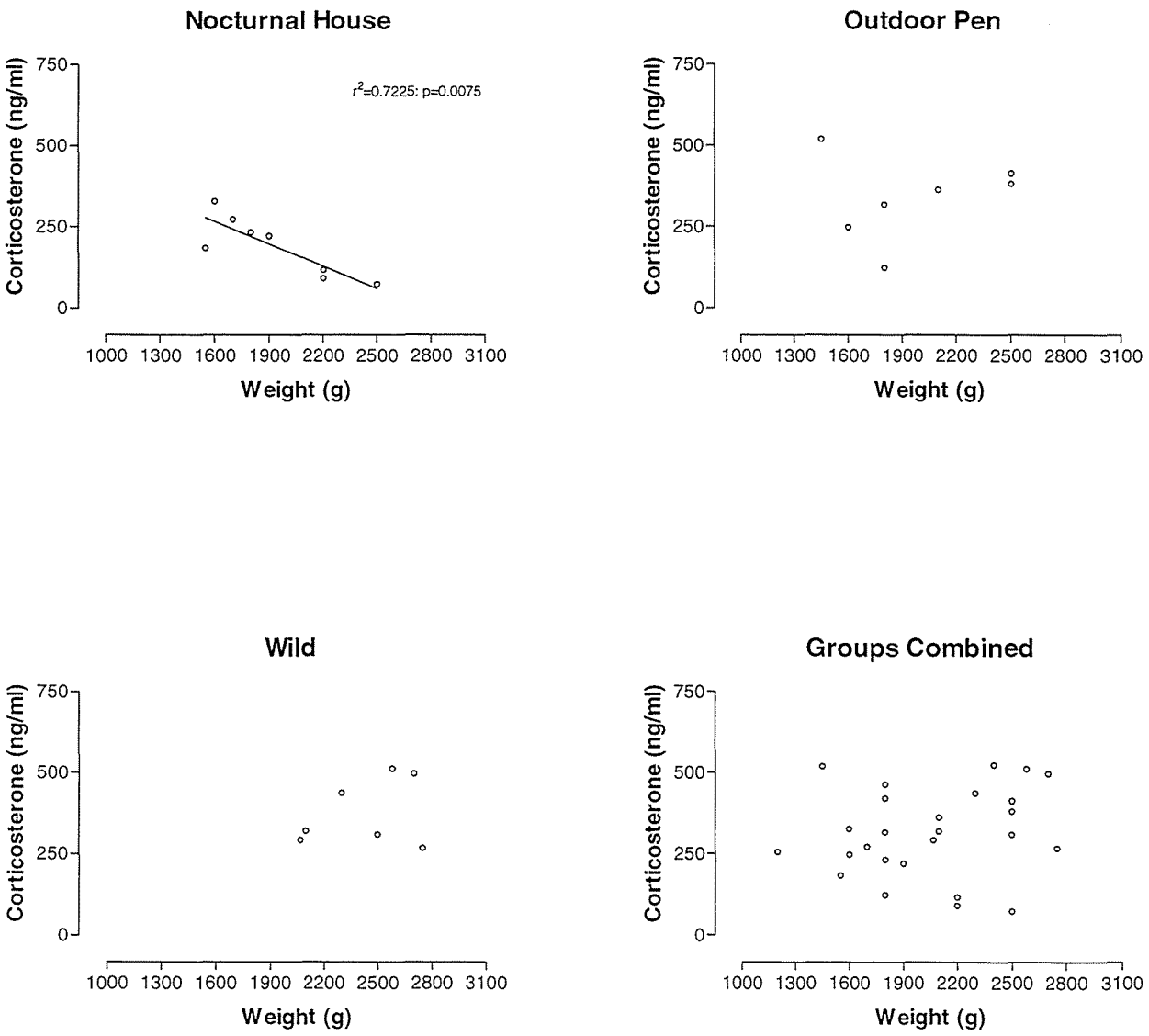


Figure 3.4.7 Correlations between body mass and corticosterone levels 30 min after capture. Significant correlations are indicated on the graphs.

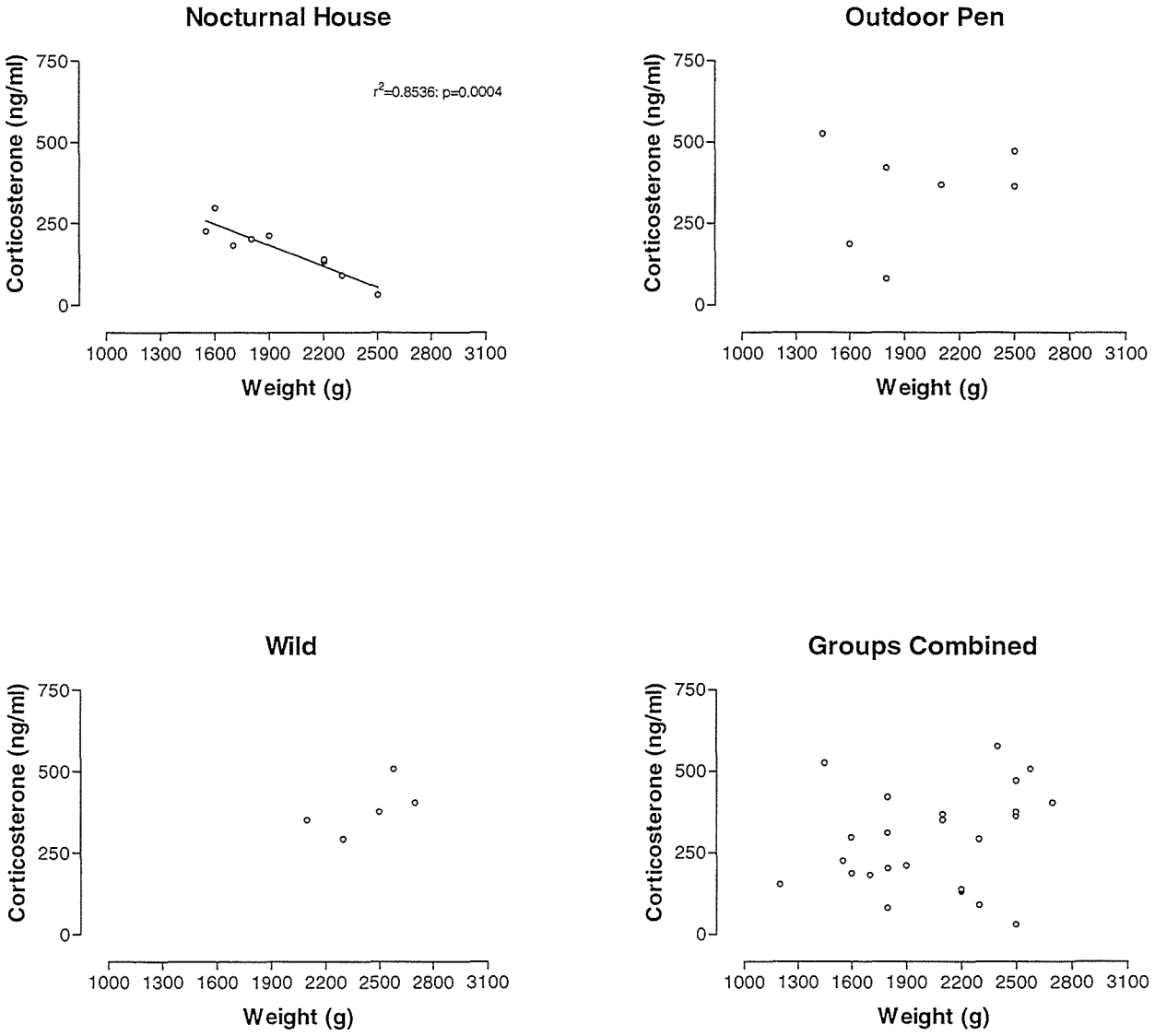


Figure 3.4.8 Correlations between body mass and corticosterone levels 60 min after capture. Significant correlations are indicated on the graphs.

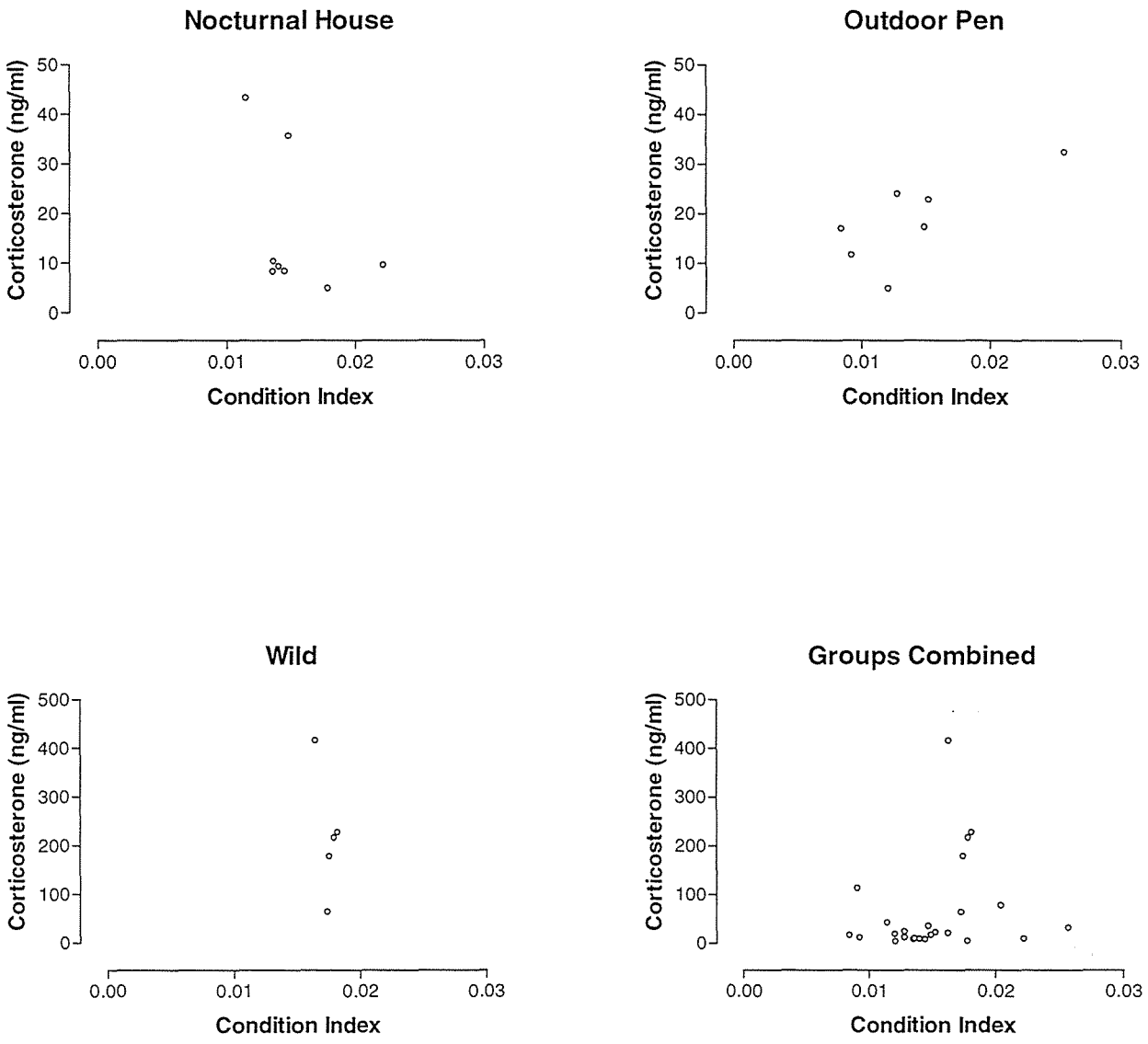


Figure 3.4.9 Relationship between condition index (body mass/toe length^3) and corticosterone levels immediately after capture. None were significant. Note the difference in y-axis scale in the lower panels.

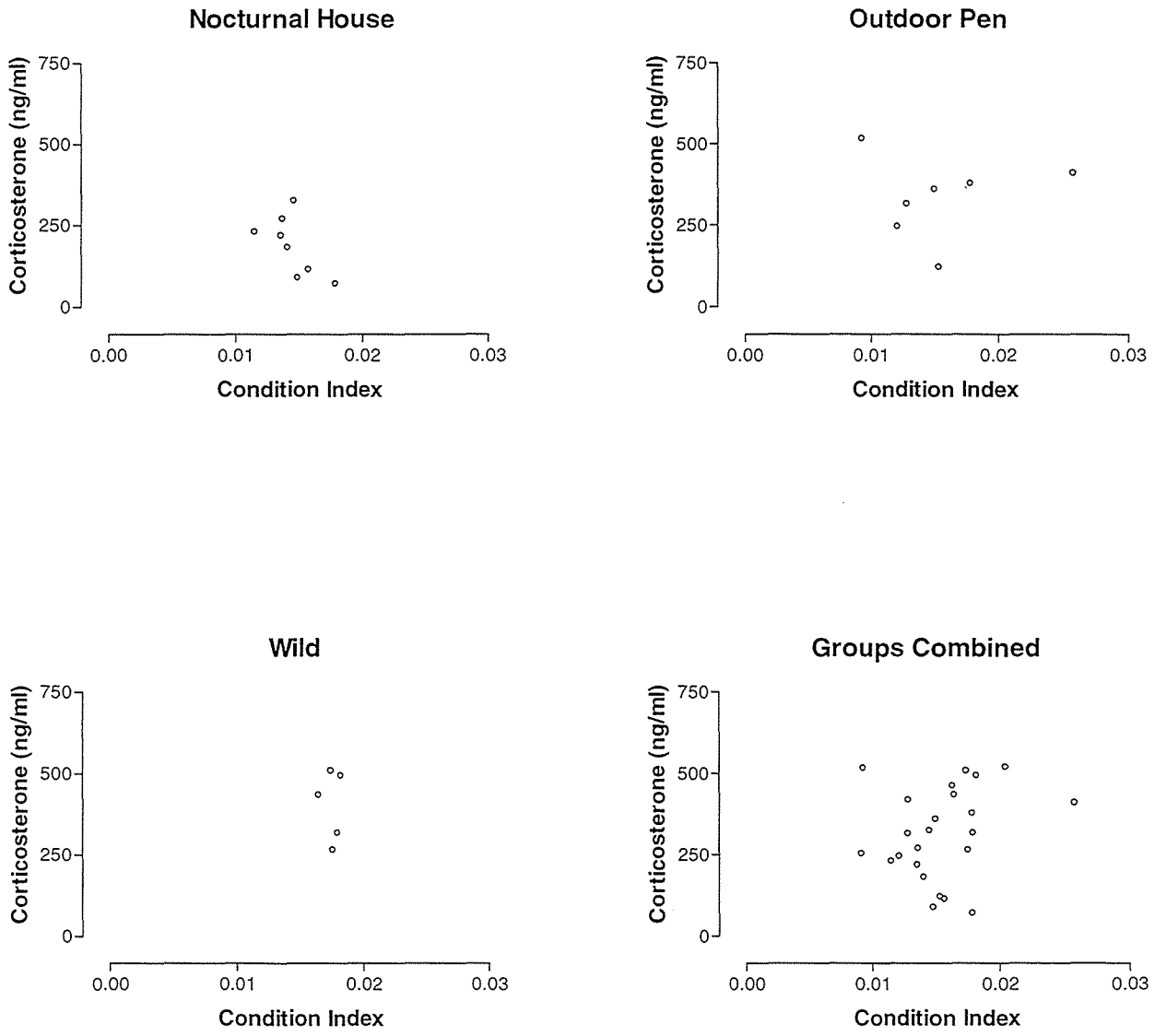


Figure 3.4.10 Relationship between condition index (body mass/toe length^3) and corticosterone levels 30 min after capture. None were significant.

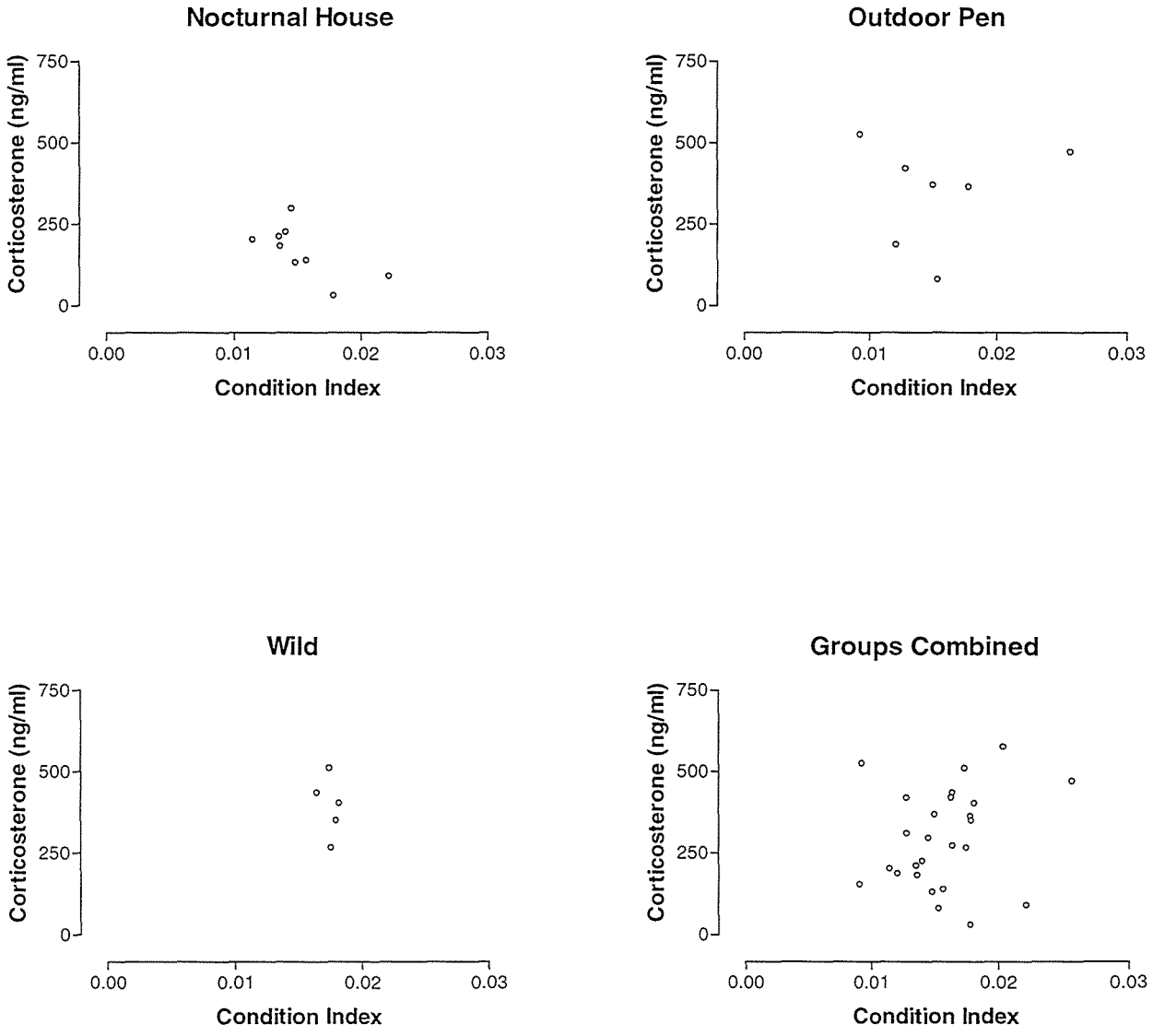


Figure 3.4.11 Relationship between condition index (body mass/toe length^3) and corticosterone levels 60 min after capture. None were significant.

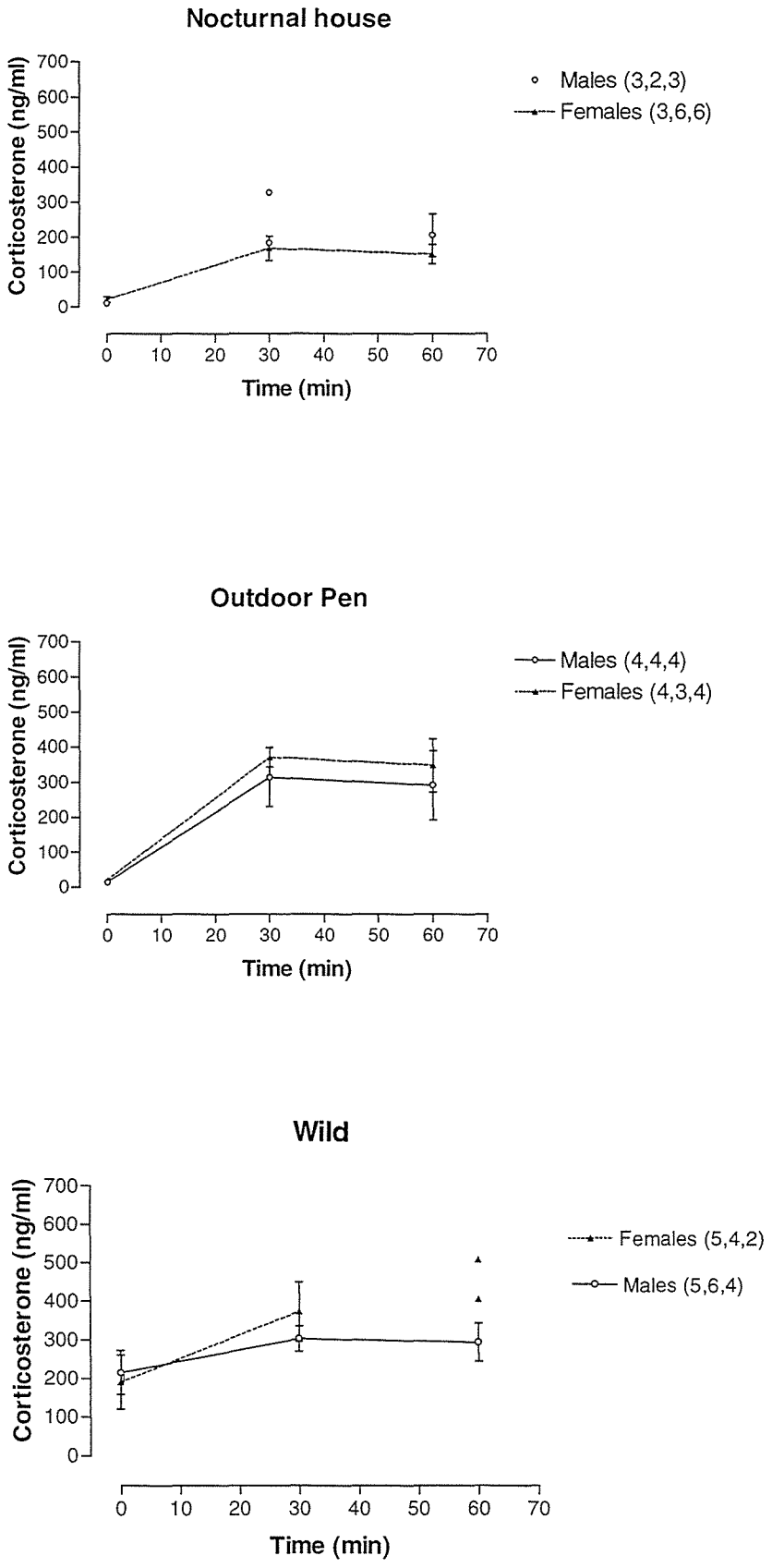


Figure 3.4.12 Changes in plasma levels of corticosterone (mean ± S.E.M.) following capture and handling, in relation to group and gender. Sample sizes are indicated in the legends.

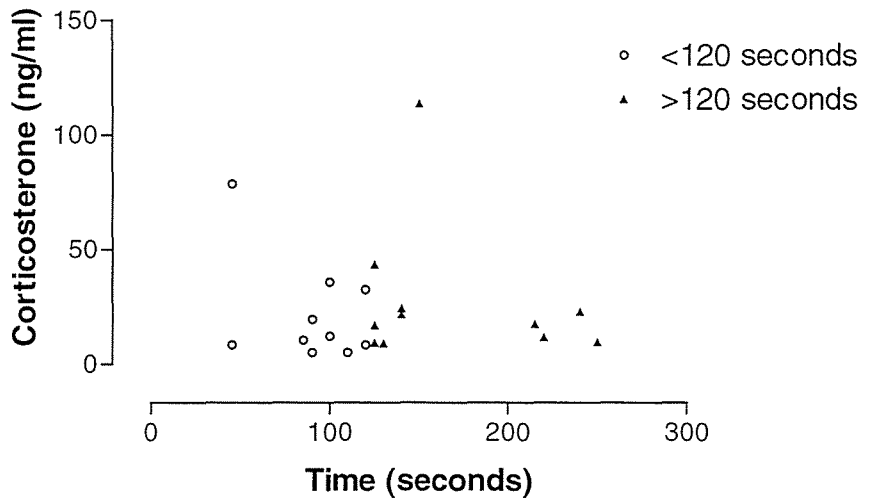


Figure 3.4.13 Time taken to collect the first blood sample within the allowed 4 min handling period. Samples divided into two groups for comparison; samples collected within 2 min (≤120 seconds) and samples collected between 2 and 4 min (>120 seconds).

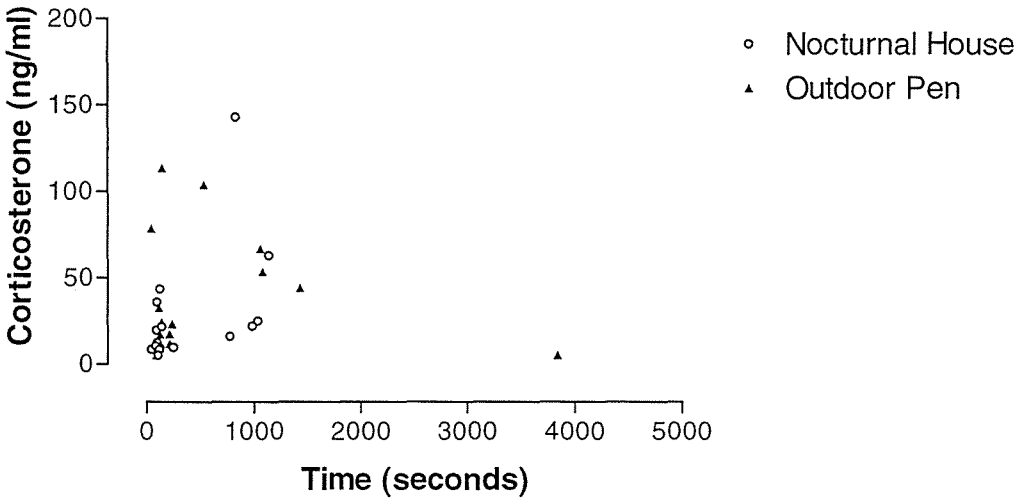


Figure 3.4.14 Relationship between plasma corticosterone concentration and the time since each captive kiwi was first disturbed.

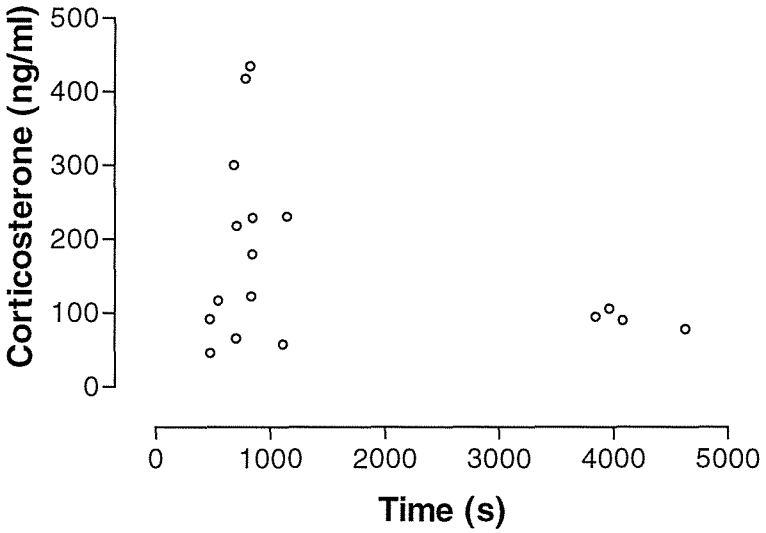


Figure 3.4.15 Relationship between plasma corticosterone concentration upon capture and the estimated time since each wild kiwi was first disturbed.

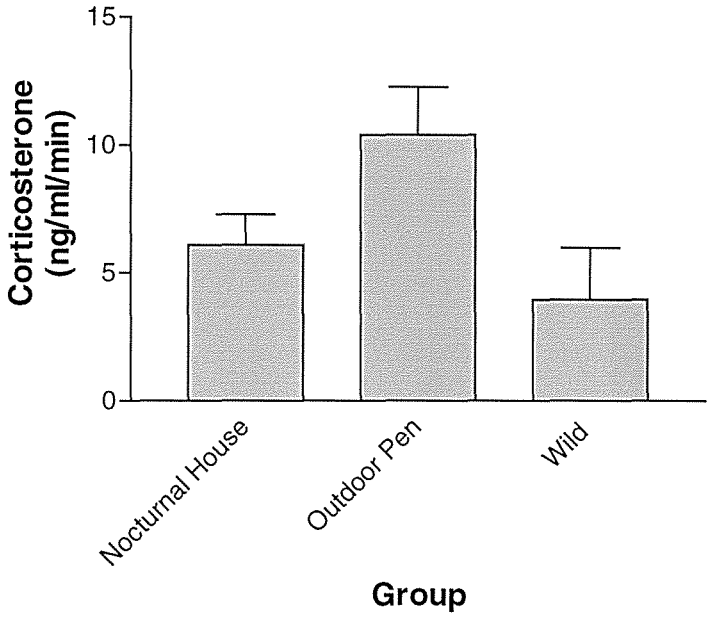


Figure 3.4.16 The mean rate (± S.E.M.) of corticosterone increase to peak levels 30 min after capture in captive (nocturnal house, outdoor pen) and wild kiwi.

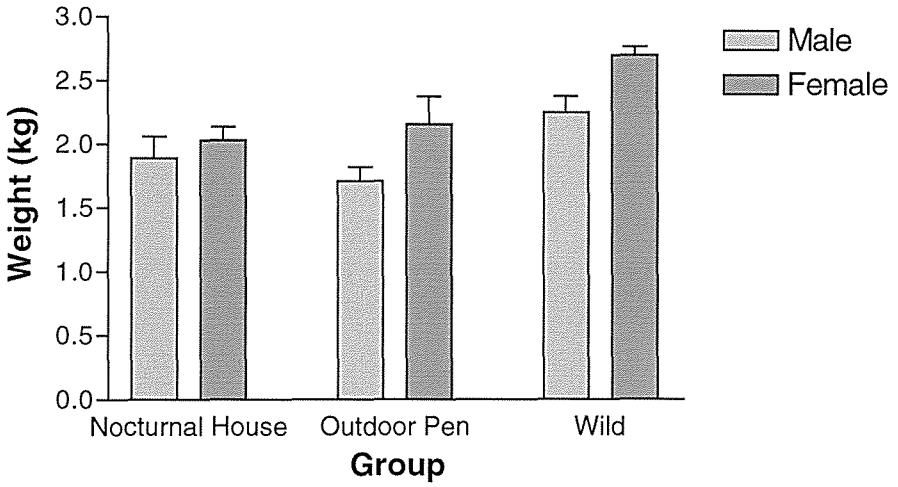


Figure 3.4.17 Comparison of body weights (mean ± S.E.M.) for each sex between captive and wild kiwi.

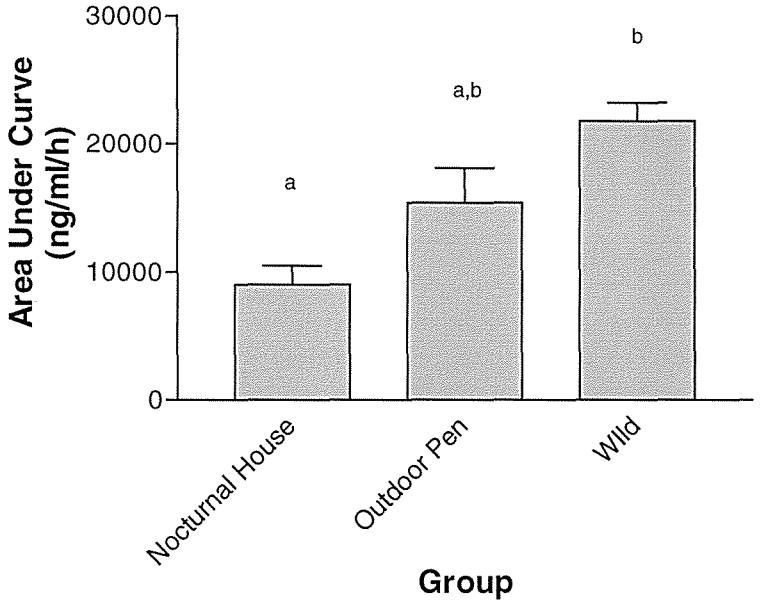


Figure 3.4.18 Mean (± S.E.M.) integrated corticosterone response (area under the curve) to capture and handling. Bars labelled with the same letter do not differ significantly.

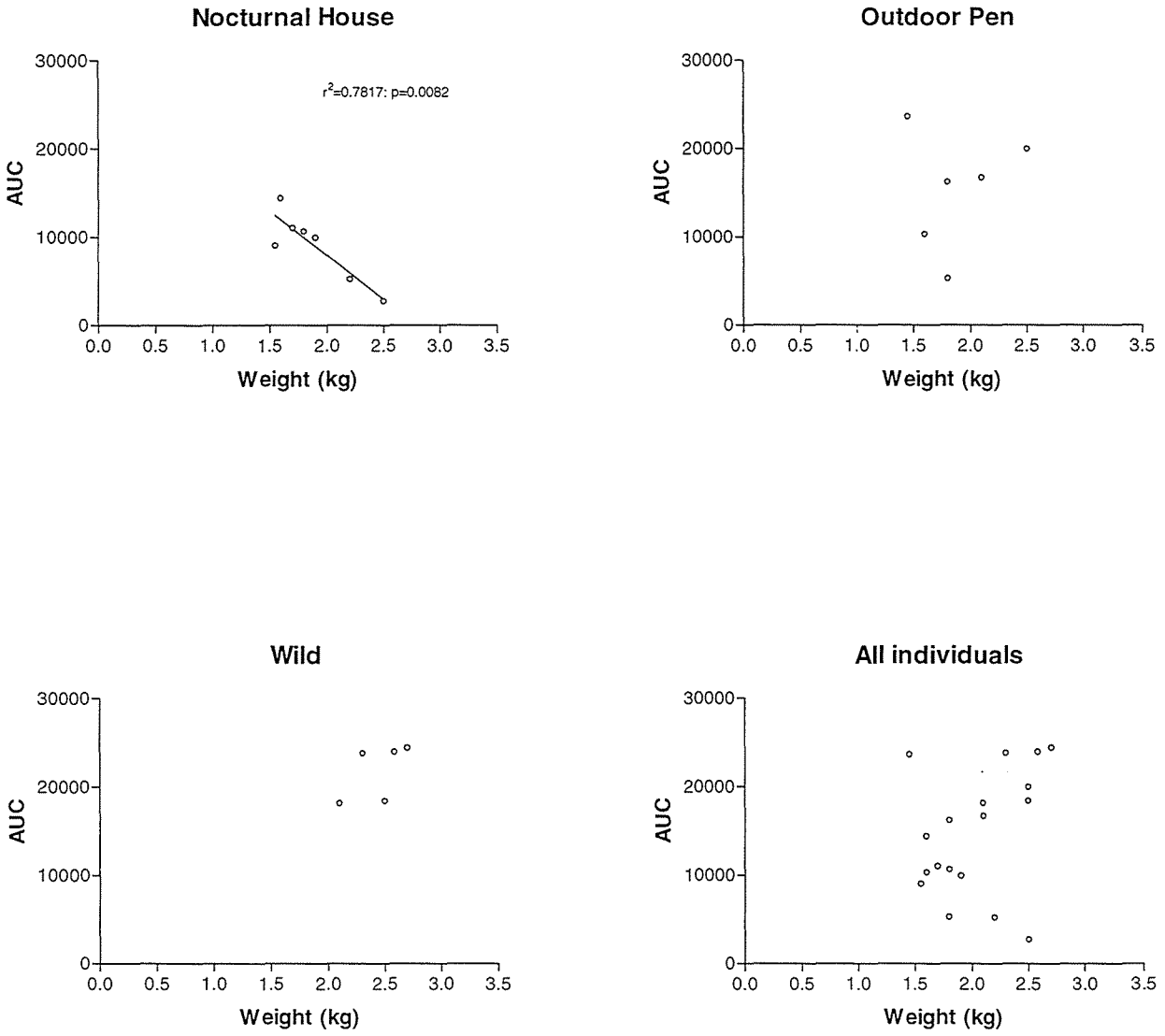


Figure 3.4.19 Relationship between body mass and the integrated corticosterone response (area under the curve: AUC, ng/ml/h). Significant correlations are indicated on the graph.

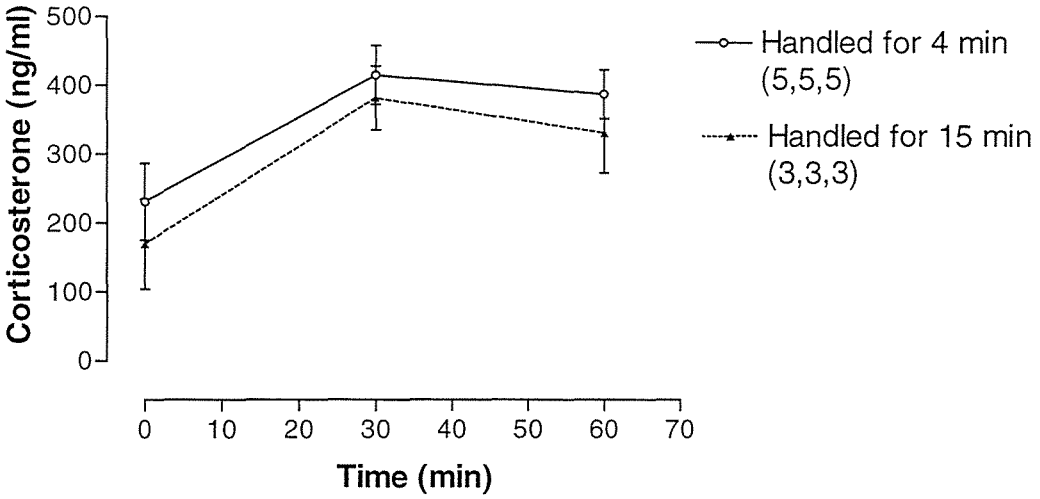


Figure 3.4.20 Changes in plasma levels of corticosterone (mean ± S.E.M.) of wild kiwi handled initially for 4 min or for 15 min. Sample sizes indicated in the legend.

Table 3.4.2 ANOVA table for comparison of plasma corticosterone concentrations between groups and at each time.

Effect	**F**	**df**	***P***
Handling duration	2.309	1,6	0.179
Time	6.788	2,12	0.011
Interaction of handling duration and time	0.035	2,12	0.966
Comparison of handling duration for each time			
Handled for 4 min vs 15 min			
0 min	0.484	1,6	0.515
30 min	0.253	1,6	0.633
60 min	0.797	1,6	0.406
Comparison of time for each handling duration			
Kiwi handled initially for 4 min			
0 min vs 30 min	6.885	1,6	0.039
0 min vs 60 min	3.042	1,6	0.132
30 min vs 30 min	0.478	1,6	0.515
Kiwi handled initially for 15 min			
0 min vs 30 min	5.491	1,6	0.058
0 min vs 60 min	1.944	1,6	0.213
30 min vs 30 min	0.952	1,6	0.367

Note: The first three rows show the results of the two-way repeat measures ANOVA for the main effects and their interaction. The remaining rows show the results of post hoc comparisons of handling duration at each time, and comparisons of times within each handling group.

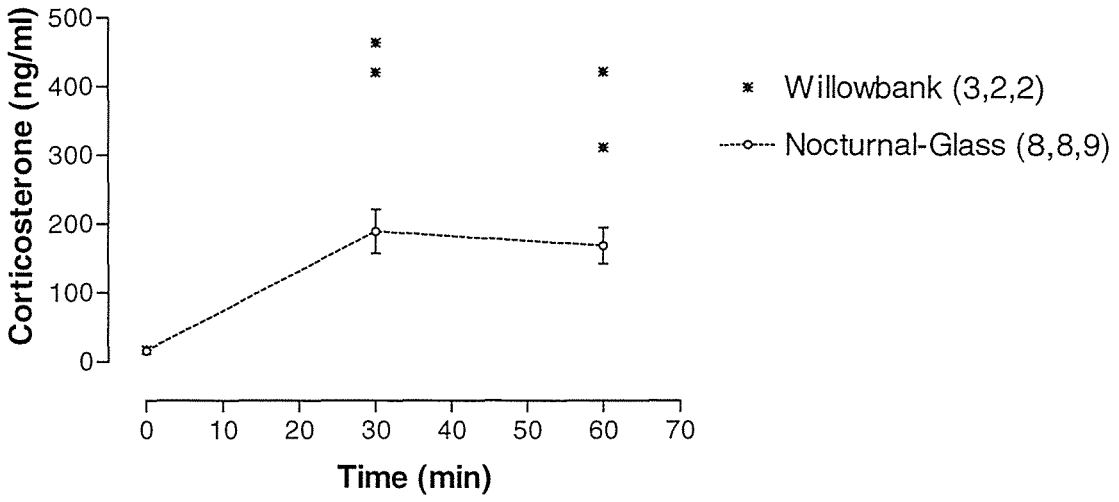


Figure 3.4.21 Changes in plasma levels of corticosterone (mean ± S.E.M.) in different nocturnal house management systems. Sample sizes indicated in the legend.

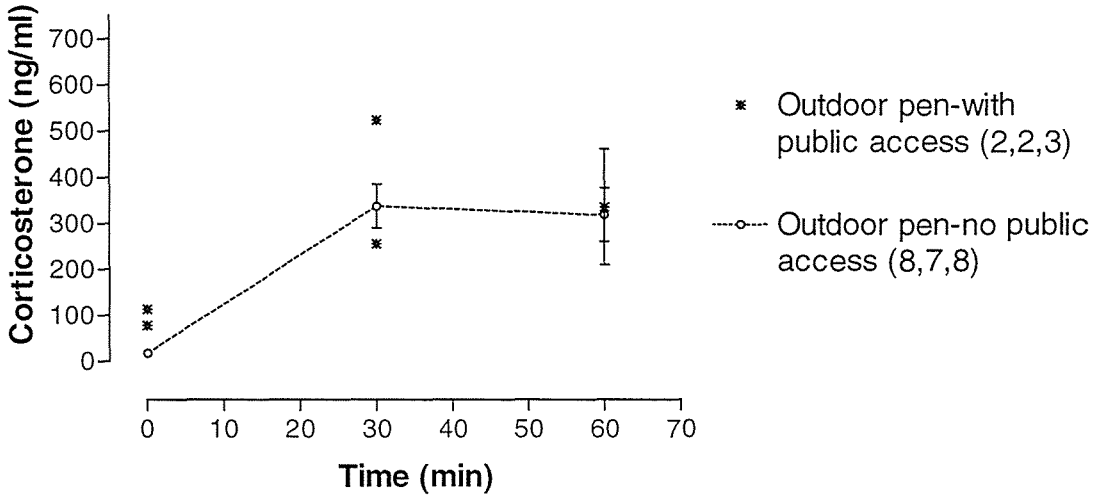
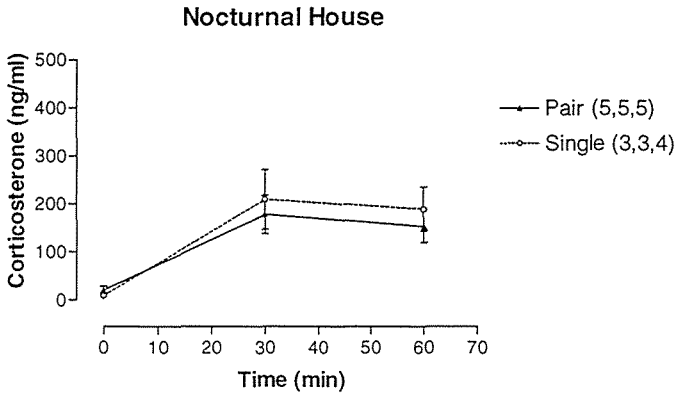
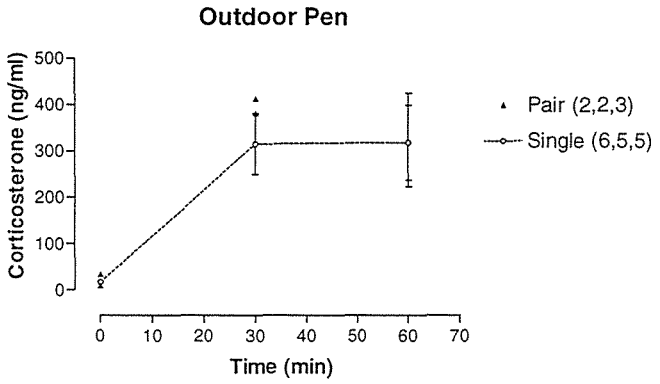


Figure 3.4.22 Changes in plasma levels of corticosterone (mean ± S.E.M.) in different outdoor pen management systems. Sample sizes indicated in the legend.





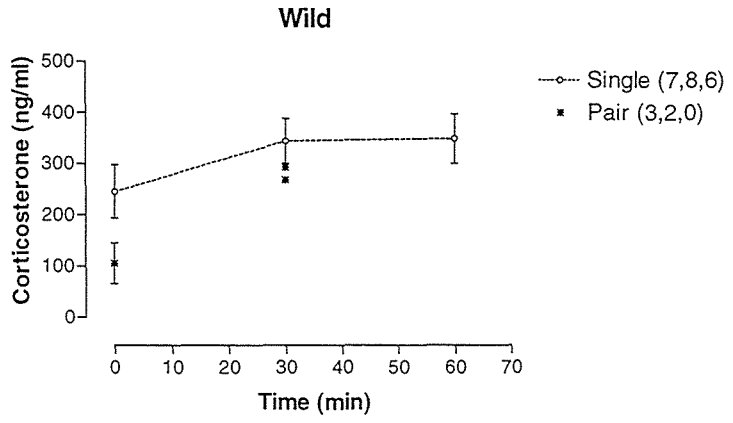


Figure 3.4.23 Comparison of the corticosterone response (mean ± S.E.M.) between kiwi housed singly, or as a pair in captivity and in the wild. Sample sizes indicated in the legends.

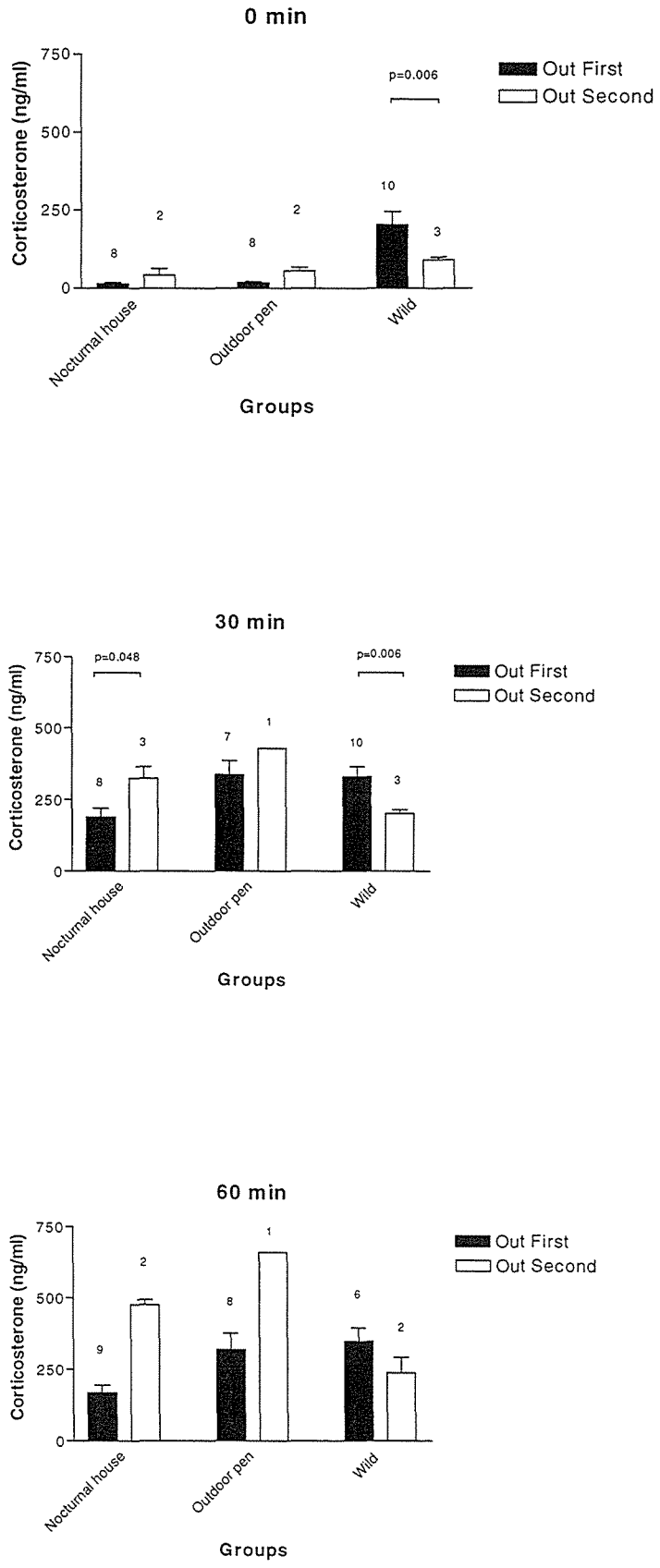


Figure 3.4.24 Comparison of corticosterone levels (mean ± S.E.M.) between kiwi removed and sampled first and those sampled second for each group. Sample sizes are indicated above each bar. Significant differences are also indicated.

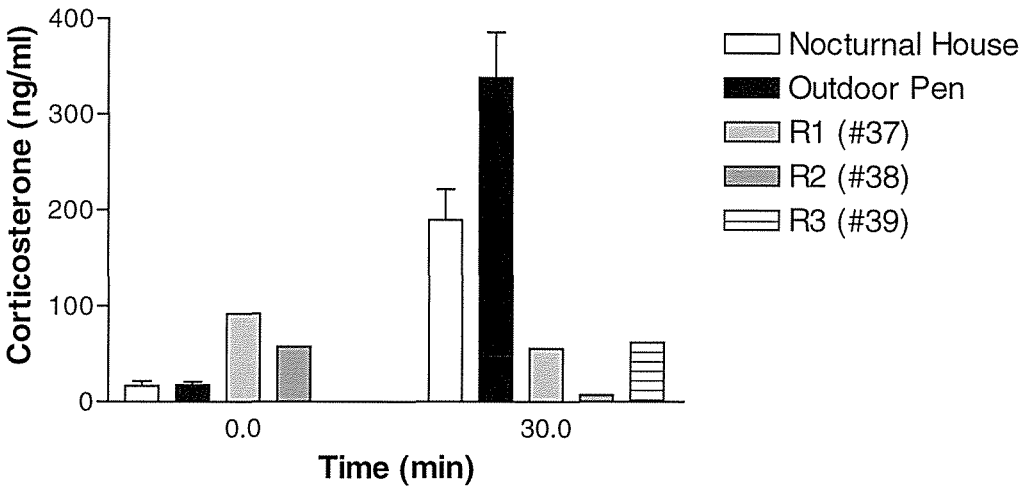


Figure 3.4.25 Individual plasma corticosterone levels of regularly handled kiwi (R1, R2, R3) before (0.0 min) and after (30.0 min) public display. Corticosterone levels (mean ± S.E.M.) of nocturnal house and outdoor pen kiwi are included for comparison.

3.5 Discussion

3.5.1 Individual variation in the corticosterone response

All kiwi responded to capture and handling with a rise in plasma levels of corticosterone. There was considerable individual variation in the corticosterone response, similar to that found in other species (for example, redpolls (*Carduelis flammea*) (Wingfield et al., 1994a), male bush warblers (*Cettia diaphone)* (Wingfield et al., 1995a), common Amakihi (*Hemignathus virens*) and Iiwi (*Vestiaria coccinea*) (Wingfield et al., 1997), male chaffinches (*Fringilla coelebs*) (Silverin, 1998b) and Magellanic penguins (*Spheniscus magellanicus*) (Fowler, 1999)).

Plasma levels of corticosterone generally peaked after 30 min and remained elevated for 60 min. This is typical of many avian species where corticosterone levels rise rapidly in response to capture and handling, and remain elevated while held captive for a short period (Astheimer et al., 1994; Smith et al., 1994; Wingfield et al., 1995a).

A negative correlation between body mass or condition index and peak corticosterone levels has been demonstrated in a few species (e.g. diving petrel (*Pelecanoides urinatrix*) (Smith et al., 1994), redpolls (Wingfield et al., 1994a), migrating bar-tailed godwits (*Limosa lapponica*) (Ramenofsky et al., 1995), fasting tan white-throated sparrows (*Zonotrichia albicollis*) (Schwabl, 1995), Magellanic penguins (Hood et al., 1998), and redstarts (*Setophaga ruticilla*) (Marra and Holberton, 1998)). However, many studies of other species have shown no correlation between body mass or condition index and peak corticosterone levels (e.g. bush warblers (Wingfield et al., 1995a), king penguins (*Aptenodytes patagonicus*) (Holberton et al., 1996), white-crowned sparrow (Romero et al., 1997), willow warblers (*Phylloscopus trochilus*) (Silverin et al., 1997), Apapane (*Himatione sanguinea*) and Iiwi (Wingfield et al., 1997), pied flycatchers (*Ficedula hypoleuca*) (Silverin and Wingfield, 1998)). It would appear that neither body mass nor condition index consistently explains the inter-individual variation in the adrenocortical response to stress in birds. In nocturnal house kiwi there was a significant negative correlation between body mass and corticosterone

levels 30 min and 60 min after capture. There was, however, no such relationship in any other group at any time period for kiwi, except for a moderate positive correlation between body mass and basal levels of corticosterone in outdoor penned kiwi. Therefore in kiwi, body mass and corticosterone appear to be generally unrelated. This is consistent with the range of results in other studies.

3.5.2 Gender and the corticosterone response

A difference between the sexes in corticosterone secretion has been recorded in some birds (Wingfield et al., 1992; Wingfield et al., 1994a; Schwabl, 1995; Hood et al., 1998), often in relation to the breeding season (Wingfield et al., 1995b). Wingfield et al., (1995b) suggest that the sex showing the greatest amount of parental care will have the lower corticosterone response to capture and handling. For example, during the breeding season, male white-crowned sparrows showed a greater increase in corticosterone in response to capture and handling than did females (Astheimer et al., 1994). This sexual dimorphism in the response was not evident during the non-breeding season (Astheimer et al., 1994), nor during the early part of the breeding season (Romero et al., 1997). Romero et al. (1997) suggest that "...the stage of the breeding season plays a major role in determining the extent of sexual dimorphism in the corticosterone response." In our study of kiwi we sampled all individuals at the same time of year before the breeding season started, thereby avoiding any possible seasonal variation that might exist between the sexes in the corticosterone response. No gender difference in either basal levels of corticosterone or the corticosterone response was evident in either captive or wild kiwi. Even though sample sizes were small and precluded statistical analysis at certain times (nocturnal house 30 min and wild kiwi 60 min samples), the distribution of the data points also suggests that there was no difference between the sexes. These results are consistent with many studies that found no differences between the sexes in either basal levels of corticosterone or their corticosterone response to capture and handling (Smith et al., 1994; Marra et al., 1995; Dufty and Belthoff, 1997; Romero et al., 1998a; Fowler, 1999). Therefore, data from both sexes were pooled for all further analyses. It would be useful in the future to sample kiwi during the breeding season, as this is the time of year when a sexual

dimorphism in the corticosterone response, if it exists at all, is most often revealed in other species (Wingfield et al., 1998).

3.5.3 Corticosterone levels immediately after capture

Plasma concentrations of corticosterone are known to rise within minutes of capture and handling (e.g. chickens (*Gallus domesticus*) (Beuving and Vonder, 1978), ducks (*Anas platyrhynchos*) (Harvey et al., 1980), white-crowned sparrows (Wingfield et al., 1982), starlings (*Sturnus vulgaris*) (Dawson and Howe, 1983), and geese (Le Maho et al., 1992)). In this study, all blood samples were collected within 4 min of capture. There was no correlation between the time taken to collect the blood sample, and plasma levels of corticosterone. Therefore, corticosterone levels found immediately after capture in captive kiwi are considered to be an accurate representation of basal levels.

Immediately after capture, captive kiwi (nocturnal house and outdoor pen) had considerably lower levels of corticosterone than did wild kiwi. Captivity has been associated with an increase in basal levels of corticosterone (in comparison to wild birds), particularly if the period of captivity is short (e.g. white throated sparrows (Marra et al., 1995) and white crowned sparrows (Romero and Wingfield, 1999)). If held captive long enough, birds may acclimatise to captivity with basal levels of corticosterone starting to approximate those found in the wild (e.g. young western screech owls (*Otus kennicottii*) (Dufty and Belthoff, 1997), black-legged kittiwake (*Rissa tridactyla*) chicks (Kitaysky et al., 1999a)). Kiwi used in this study had been in captivity for a considerable period prior to sampling (from 1 yr to 20+ yr). It is possible that the low basal levels of corticosterone are a result of acclimatisation to long periods in captivity.

Elevated basal levels of corticosterone can be associated with the breeding season (Romero and Wingfield, 1998; Romero and Wingfield, 1999), and food restriction (Hood et al., 1998; Kitaysky et al., 1999a). Similar factors may be responsible for the elevated corticosterone levels found immediately after capture in wild kiwi. However this is unlikely as we sampled wild and outdoor pen kiwi during the same season, thus removing any effect (if it existed at all) season may have had on the sensitivity of the

HPA axis. Wild kiwi are also free to move and hunt for food at will (in captivity they are not), and are on average larger than captive birds. It is therefore unlikely that food restriction explains the elevated plasma levels of corticosterone in wild kiwi. It is most likely that the elevated corticosterone levels found in wild kiwi are a result of our sampling methods. Catching and blood sampling a kiwi in captivity can be completed within seconds. Therefore, corticosterone levels immediately after capture probably reflect true basal levels. In the wild, however, capture of kiwi using radio-telemetry gear can be difficult. Consequently, wild kiwi are likely to be aware of our presence some 5 to 18 min prior to their capture. Therefore a corticosterone response may have been initiated before capture, accounting for the elevated levels found immediately after capture. In addition, the lack of a significant rise in corticosterone 30 min after capture suggests that a corticosterone response was induced before capture in wild kiwi. Similarly, during a severe storm, diving petrels were found to have significantly elevated levels of corticosterone immediately after capture (Smith et al., 1994). Levels remained elevated during the 60 min of capture and handling. This led Smith et al. (1994) to suggest that a maximal corticosterone response was already initiated prior to capture in diving petrels. Interestingly, there was no correlation between the estimated time taken to catch wild kiwi and plasma corticosterone concentrations when caught. However, there may have been a relationship had we been able to accurately determine the time between kiwi becoming aware of our presence and when they were caught and sampled.

Elevated corticosterone levels have been shown to redirect behaviour and physiology away from reproduction towards immediate survival in birds (for reviews see Wingfield et al., 1997; Silverin, 1998b; Wingfield et al., 1998; Carsia and Harvey, 2000). These results indicate that merely searching for the position of a kiwi can initiate a significant rise in plasma levels of corticosterone. Northern brown kiwi are known to be very sensitive to disturbance during the early part of incubation. When disturbed, male kiwi will often abandon the nest. This sensitivity to disturbance is believed to vary between seasons, with male kiwi becoming increasingly tolerant to disturbance during the later parts of incubation (J. McLennan pers. comm.). Therefore, searching for wild kiwi during routine management practices (disturbance) could cause an elevation in plasma levels of corticosterone, as observed in this study.

However, it is important to bear in mind that this experiment was conducted during the non-breeding season. It is entirely possible that kiwi may down regulate the sensitivity of the HPA axis to stress during the breeding season, as has been demonstrated in some species that breed in severe environments (see Wingfield et al., 1997 and Silverin, 1998b for reviews). Blood sampling at different times of the year would indicate whether the increasing tolerance of incubating male kiwi to disturbance is associated with a decrease in the corticosterone response during the later part of incubation.

3.5.4 Corticosterone response to capture and handling

The reduced corticosterone response to capture and handling of nocturnal house kiwi compared with wild kiwi might be explained by several physiological or ecological conditions, including, habituation to humans, a seasonal cycle in the corticosterone response, or perhaps limited food quality and availability. Each of these hypotheses will be discussed here.

A reduction in the corticosterone response to human disturbance has been demonstrated in avian species exposed to high levels of human contact. For example, daily handling of captive American kestrels (*Falco sparverius*) may have caused the decline in their corticosterone levels (Heath and Dufty, 1998). Also, postnatal handling of Amazon parrots (*Amazona amazonica*) lowers their corticosterone response to further handling, suggesting that they do not perceive handling as stressful (Collette et al., 2000), as does regular contact with humans in broilers (Hemsworth et al., 1994). Interestingly, domestic fowl chicks allowed to observe the handling of other chicks, or having a human stand near their cage was just as effective as regular handling in reducing fear of humans (Jones, 1993). Likewise, nesting Magellanic penguins exposed to very high levels of human visitation (specifically tourist) do not initiate a corticosterone response when disturbed (person standing 1 m from the bird for 5 min) (Fowler, 1999). However, if the nesting penguins had been visited for only a short period each day (by a researcher), then they do respond to a similar disturbance with an increase in corticosterone. It appears then, as Barnett et al., (1994) also suggested, that both the quality and quantity of handling is important in reducing a bird's adrenocortical response to handling. Even though kiwi in a nocturnal house are exposed to humans

more regularly than are wild or outdoor pen kiwi, the level of human interaction is limited compared to the species mentioned earlier. Kiwi held in nocturnal houses are exposed to humans only during routine husbandry or through glass-fronted enclosures (which allow only a limited amount of aural and visual interaction with the public). It is questionable then whether the minimal amount of human contact experienced by nocturnal house kiwi would be of sufficient quantity or quality to cause a reduction in the adrenocortical response to the novel stimulus, capture and handling.

It is interesting to note that nocturnal house kiwi may not be responding to capture and handling with a maximal release of corticosterone. Nocturnal house kiwi removed from their burrows second had already been disturbed when their mate was removed and sampled earlier. This increased amount of disturbance was associated with significantly elevated plasma corticosterone levels 30 min after capture. This trend was not repeated in outdoor pen kiwi, where there appears to be no difference between kiwi removed first and kiwi removed second (i.e. a maximal corticosterone response by kiwi removed first and second). It appears that only with repeated disturbance does the acute rise in corticosterone levels of nocturnal house kiwi reach those of outdoor pen or wild kiwi. This may suggest that capture and handling was not perceived as being equally stressful by all groups of kiwi.

The sensitivity of the HPA axis to stress has been shown to fluctuate throughout the annual cycle in wild birds (for examples see Wingfield et al., 1992; Astheimer et al., 1994; Wingfield et al., 1994a; Holberton et al., 1996; Romero et al., 1997; Silverin, 1997; Romero and Wingfield, 1998; Romero et al., 1998b; Silverin, 1998a; Silverin, 1998b; Kitaysky et al., 1999b). This seasonal variation in the corticosterone response may also persist in captivity. For example, Holberton (1999) showed that a reduction in the stress response, concurrent with migratory fattening, was displayed in yellow-rumped warblers (*Dendroica coronata*) that were held in captivity with a seasonal light cycle, even in the absence of other environmental stimuli. Romero and Remage-Healey (2000) also found that a seasonal variation in basal and stress-induced corticosterone levels (lowest during moult) found in wild starlings persists in captivity.

In contrast, an increase in sensitivity to acute stress during the breeding season in wild white-crowned sparrows (Romero et al., 1997) was not reflected in captive birds that

were held under natural light conditions at the same time of year (Romero and Wingfield, 1999). Breuner et al. (1999) also showed that the corticosterone response of captive photostimulated and photosensitive white-crowned sparrows differed to their wild counterparts (see Romero et al., 1997 in Breuner et al., 1999; Romero and Wingfield, 1999). Similarly, seasonal variation in the corticosterone response of wild Lapland longspurs (*Calcarius lapponicus*) was not demonstrated by captive conspecifics (Romero et al., 1998c). In general then, captive species do not always display seasonal variations in the corticosterone response, as demonstrated by their wild counterparts. Furthermore, these results suggest that captivity *per se* may have a profound effect on the functioning of the HPA axis.

Wild kiwi, as seen in many other species, may exhibit seasonal variation in the corticosterone response. It is therefore possible that the corticosterone response in wild and outdoor pen kiwi might be lower or higher at a different time of year. Nocturnal house kiwi are held under an unchanging photoperiod, which differs to that of outdoor pen and wild kiwi at that time of year. This might at least partly contribute to the differences in their corticosterone response. However, this study only compared corticosterone responses at one time of year. Sampling kiwi at different times of year would be necessary to explore this hypothesis further.

If the lower corticosterone response of kiwi housed in the nocturnal house is related to a non-seasonal photoperiod, then it is likely that other endogenous rhythms such as the reproductive cycle would also be affected. This may help explain the reduced breeding success of kiwi housed in nocturnal houses (M. Potter pers. comm.), given that kiwi are essentially short day breeders, and that nocturnal house kiwi are constantly held on a long day photoperiod. This could be investigated further by comparing the annual cycle of gonadal steroids (perhaps via a non-invasive faecal steroid analysis) in captive kiwi with those already established for wild kiwi by Potter and Cockrem (1992).

Although the pattern of corticosterone release may be under endogenous control (as a function of the annual cycle), it is likely to be influenced by exogenous factors such as food quality and availability. In captive American kestrel's fed ad libitum, corticosterone returned to basal levels within an hour of being captured (Heath and Dufty, 1998). However, corticosterone levels remained elevated after an hour in

kestrels held on caloric depleted diets. Also, both basal levels and the corticosterone response were elevated in black-legged kittiwake chicks feed on caloric depleted diets (Kitaysky et al., 1999a). Furthermore, chickens held on a protein restricted diet showed a four fold increase in the corticosterone secretion rate (Carsia et al., 1988). Therefore, diet quality can influence the corticosterone response of birds. Nocturnal house kiwi rely solely on an artificial diet for their nutritional requirements, with essentially no access to natural prey items (invertebrates). This artificial feed is, however, of questionable nutritional quality (M. Potter pers comm). Outdoor penned kiwi, on the other hand, have access to a limited natural supply of invertebrates as well as the artificial diet. The diet of outdoor penned kiwi is likely then to be of better quality than that of nocturnal house kiwi. If the diet of nocturnal house kiwi was inadequate, then we would expect that basal corticosterone levels would be higher, the corticosterone response would be elevated, and that these levels would remain elevated longer compared to outdoor pen kiwi. However, nocturnal house kiwi had similar basal corticosterone levels to outdoor pen kiwi, and a lower adrenocortical response to capture and handling than both outdoor pen and wild kiwi. Plasma corticosterone levels also returned quickly to basal levels in nocturnal house kiwi 3 h after capture. Therefore, either nocturnal house kiwi do not have an inadequate (caloric depleted) diet, or dietary deficiencies do not affect corticosterone levels in kiwi the way it does in some other birds.

Food availability may also affect the corticosterone response (Carsia and Harvey, 2000). An inverse relationship between fat stores and the stress response in some avian species appears during periods of limited food availability, for example snow buntings (*Plectrophenax nivalis*) and Lapland longspurs (Wingfield et al., 1994b), and breeding redpolls (Wingfield et al., 1994a). This relationship may not exist in species with unlimited food supply (e.g. Florida scrub-jay (*Aphelocoma coerulescens*) (Schoech et al., 1997)), or in species with a priority on storage of protein (Heath and Dufty, 1998). Therefore, the negative correlation between maximum corticosterone levels and body weight of nocturnal house kiwi may also be a result of limited food availability. However, because we measured body mass and not fat score, this link is a tenuous one.

It is unclear whether a steep or shallow rise in the corticosterone response is characteristic of poor body conditio. For example, Wingfield et al. (1997) suggest that a

steep rise in corticosterone is related to poor body condition. Conversely, Heath and Dufty (1998) found that food-restricted birds took significantly longer to reach maximum corticosterone levels than birds feed ad libitum. The rate of increase in corticosterone did not differ between nocturnal house and outdoor penned kiwi. Therefore, body condition is unlikely to differ between nocturnal house and outdoor penned kiwi, which is consistent with the lack of a significant difference in body mass between these two groups. Other nutritional parameters that may limit body size, but not cause a variation in the corticosterone response, might explain this difference. Further investigation into the nutritional content of the diet for captive kiwi is required to explain the difference in body weights.

In conclusion, habituation and seasonal variation may independently or in concert explain the reduced sensitivity of the HPA axis in nocturnal house kiwi. It is not possible to distinguish between these without further investigation.

3.5.5 The integrated corticosterone response

The integrated corticosterone response (area under the curve, AUC) represents a broader measure of the adrenocortical response to stress, as it includes both the rate of corticosterone increase and the rate of corticosterone clearance (Breuner et al., 1999). Comparison of the AUC between groups revealed identical trends to those already described when comparing the corticosterone response. Kiwi held in the nocturnal house had significantly less circulating corticosterone than those of wild kiwi, but not less than outdoor penned kiwi. There was also no difference between outdoor penned and wild kiwi.

3.5.6 The effect of handling duration

The duration of handling has been shown to significantly affect the magnitude of the corticosterone response in blackbirds (*Terdus merula*) (M. Welch, unpublished data). Blackbirds handled for 2 or 4 min immediately after capture had a significantly lower corticosterone response than those handled for 6 or 8 min. Similarly, male mule ducks

(cross between male *Cairina moschata*, and female *Anas platyrhynchos*) immobilised for 30 sec and blood sampled (occipital sinus puncture) had significantly lower corticosterone levels 15 min after capture than those handled initially for 90-150 sec (wing vein puncture, which requires more handling) (Noirault et al., 1999). In wild kiwi, however, there was no effect of handling duration (4 min compared with 15 min) on the corticosterone response. As already mentioned, it is likely that an adrenocortical response to stress was initiated some time prior to capture in wild kiwi. If plasma corticosterone levels were elevated prior to capture (as suggested by the data), then it is unlikely that we would detect an increase in the corticosterone response due to increased handling 30 min after capture. Similarly, diving petrels were found to have elevated corticosterone levels when captured during a severe storm (Smith et al., 1994). Corticosterone levels failed to increase further during capture and handling, due to the petrels being stressed at the time of capture. These results do not preclude the possibility that duration of handling may influence the magnitude of the corticosterone response in kiwi. It is likely that the elevated corticosterone levels upon capture masked any effect that handling duration may have had on the corticosterone response.

Handling duration may not only change the magnitude of the corticosterone response, it may also have an effect on the duration of the corticosterone response. To date, there have been no studies that have addressed this question. Although we now know what effect disturbance, capture and handling have on the corticosterone response of wild kiwi, we do not know whether corticosterone levels remain elevated for extended periods after prolonged disturbance. Given the negative effects that chronically elevated levels of corticosterone can have (see Wingfield et al., 1998 for review), a study such as this has important implications for the management of kiwi, and for other avian species.

3.5.7 Comparison of captive management systems

Typically, a nocturnal house enclosure is constructed with a glass front so that the public can view the kiwi without disturbing them. There is, however, a second type of nocturnal house, where the public walks through the enclosure and are separated from the birds by a low wooden fence (at Willowbank, Christchurch, NZ). Basal levels of

corticosterone did not differ between kiwi in these two types of enclosure. Due to small sample sizes, statistical analysis of the data at 30 min and 60 min after capture was not possible. However, the corticosterone response tended to be higher in kiwi held at Willowbank compared to those held in a typical nocturnal house enclosure. With such small sample sizes we can only speculate as to whether this is a true trend or an artefact of sample size. Also, there tended to be no difference in levels of corticosterone at any time between the two types of outdoor pen management systems, those with or without public access close to the pen. Again, small sample sizes precluded statistical analysis immediately after, or 30 min after, capture. Therefore we can still only speculate about the significance of these results. Larger sample sizes are necessary to investigate what effect (if any) the Willowbank nocturnal house management system has on the sensitivity of the HPA axis. Similarly, further work investigating the effect of increased disturbance at outdoor pens is necessary.

Kiwi in New Zealand are held in captivity either singly or as pairs (intended for breeding). We found that there was no difference between those held singly or as a pair in either basal levels of corticosterone or the corticosterone response at any time in any group. Also, plasma corticosterone levels immediately after capture in wild kiwi were similar between those found alone or with a mate. Where small samples sizes did not allow statistical analysis, data tended to be similar between those held (or found) alone or as a pair. While small sample sizes preclude any rigorous conclusions from these data, the data suggest that the adrenocortical response to stress is not affected by whether kiwi roost alone or with a mate.

3.5.8 The corticosterone response of regularly handled kiwi

We have established that all kiwi respond to a novel stimulus (capture and handling) with an increase in plasma concentrations of corticosterone. In contrast, plasma levels of corticosterone in regularly handled kiwi *did not* increase after 30 min of public display, although due to small sample sizes, statistical analysis was not possible. Furthermore, the corticosterone levels 30 min after kiwi were first brought out for display were lower than plasma levels in captive kiwi (unaccustomed to handling) 30 min after capture and handling.

In several avian species, regular human contact has been shown to reduce fear of humans (Jones, 1993; Barnett et al., 1994; Hemsworth et al., 1994), increase feed conversion (Hemsworth et al., 1994) and lower the corticosterone response to handling. For example, regular handling (for 15 min per day, 5 days per week) is enough to reduce the adrenocortical response to handling in laying hens (Barnett et al., 1994). The type of handling in broilers also seems important, where gentle handling in an upright position results in lower plasma levels of corticosterone than those handled in an inverted position (Kannan and Mench, 1996; Kannan and Mench, 1997). Neonatal handling may also attenuate the adrenocortical response to handling, as seen in domestic fowl (Hemsworth et al., 1994), Amazon parrots (Collette et al., 2000) and perhaps (but not conclusively) in young western screech-owls (Dufty and Belthoff, 1997). Conversely, Kannan and Mench (1997) found that the corticosterone response to handling at an older age could not be markedly reduced by handling during rearing in broilers. Also, handling of domestic hens that are unaccustomed to the handling procedure can result in reduced egg production, and an increase in the number of equatorial bulges and cracks (Hughes and Black, 1976). It is apparent that the quality, quantity and timing of handling is important, if it is to produce marked (positive) changes in a bird's response to handling. It is obvious though that humans can habituate avian species to handling. Our results agree with those of these earlier studies and indicate that kiwi can (and have) become accustomed to handling for public display.

The mechanisms underlying this apparent habituation to handling are poorly understood. One hypothesis is that the reduced response is caused by increasing the sensory threshold required to elicit a response (i.e. psychological coping) (Harvey et al., 1984). It is possible then that due to prior experience (expectation of events) the handling stimulus is no longer deemed stressful by the individual. However, the avian adrenal gland is also capable of becoming desensitised to high and prolonged exposure to exogenous ACTH (Kalliecharan, 1981; Davison et al., 1985). Therefore, any apparent reduction in corticosterone levels in regularly handled kiwi may also be caused by a down regulation of the adrenocortical responsiveness to corticosterone secretagogs, and not because of any change in the individual's perception of the stimulus.

Corticosterone levels in two regularly handled kiwi, measured immediately before a public display session, were higher than mean basal levels found in non-regularly handled captive kiwi. With such small sample sizes it is impossible to say if this is a true trend for regularly handled kiwi. However the results do suggest that this is an important area for further investigation, perhaps by measuring corticosterone in the droppings of regularly handled kiwi over a longer period of time.

3.5.9 Conclusions

Kiwi, as with all avian species that have been studied, respond to capture and handling with an increase in corticosterone. Although there is considerable variation in the response, there appears to be no difference between the sexes. In addition, the corticosterone response appears to be generally unrelated to body mass or condition index.

Corticosterone levels found immediately after capture were significantly higher in wild kiwi than in captive kiwi. This was most likely due to the method of capture, as wild kiwi took considerably longer to catch than captive kiwi. The low basal levels of corticosterone in captive kiwi do suggest, however, that they have become acclimatised to captivity.

Nocturnal house kiwi had a lower corticosterone response to capture and handling than wild kiwi. The cause of this difference is unclear. It is possible that nocturnal house kiwi have become habituated to the presence of humans. Alternatively, a possible seasonal variation in the adrenocortical response may also explain the difference. Furthermore, it is conceivable that both factors are acting together to produce the reduced corticosterone response of nocturnal house kiwi. Importantly, the lack of significantly elevated levels of corticosterone in captive kiwi indicates that there is no increase in the sensitivity of the HPA axis to stressors in captive kiwi.

Handling duration appears to have had no effect on the corticosterone response of wild kiwi. However, the elevated corticosterone levels found in wild kiwi upon capture most

likely masked any effect handling duration may have had on the corticosterone response.

Varying captive management systems appear to have little effect on either basal levels of corticosterone or the corticosterone response. Nocturnal house enclosures with increased aural and visual interaction between kiwi and the public also appear to have little influence on corticosterone responses. Likewise, increased public access close to outdoor pens has a similar (lack) of effect on the adrenocortical response. However, small sample sizes precluded a detailed analysis of these management systems. Further work is required to refine these results.

Regularly handled kiwi appear not to respond to handling and public display with an adrenocortical response. This may indicate that kiwi have become habituated to handling in this manner. Alternatively, it may reflect a reduced adrenocortical sensitivity to ACTH. In addition, elevated basal levels may suggest a chronic elevation in corticosterone release. This could be further investigated with a long term, non-invasive study of faecal corticosterone concentrations.

4 Corticosteroid binding globulin

4.1 Abstract

It is generally accepted that only free corticosterone, that which is not bound to corticosterone binding globulin (CBG), has a biological influence on target cells. A series of experiments were conducted to determine the optimal conditions and concentrations of competing ligands to characterise CBG in the plasma of Japanese quail (*Coturnix coturnix japonica*) and kiwi (*Apteryx mantelli*). Increasing the temperature during the stripping of endogenous steroid from the plasma of quail had no effect on the amount of corticosterone removed. Furthermore, a higher temperature (36°C) resulted in a significant reduction in CBG concentration. Conversely, the increased temperature during stripping of kiwi plasma resulted in a significant increase in the amount of corticosterone removed (60% at 4°C, 93% at 36°C). Increasing the temperature had no effect on the binding affinity (K_d) and capacity (B_{max}) of CBG. The best conditions for stripping quail plasma is by adding a dextran-coated charcoal (DCC) buffer solution directly into plasma at 20°C for 2 h. For kiwi, the best conditions were 36°C for 1 h with a DCC-buffer solution added directly to the plasma. The optimum concentration of cold and amount of hot ligand to determine K_d and B_{max} of quail CBG were 100 ng/ml and 10 000 cpm. For kiwi these concentrations were 1 000 ng/ml and 10 000 cpm. There was no significant difference in B_{max} between kiwi in the nocturnal house (1356 ± 98 nM), outdoor pen (1168 ± 170 nM) or wild (1517 ± 44 nM). There was also no significant difference in K_d between kiwi in the nocturnal house (2947 ± 182 nM), outdoor pen (3056 ± 408 nM) or wild (2506 ± 153 nM). There were differences between Scatchard and non-linear regression analysis in their determination of K_d, but not of B_{max}. Maximum corticosterone titres did not exceed B_{max} at any time for any group of kiwi. Therefore the binding capacity of CBG was not considered to be a major factor when interpreting corticosterone responses of kiwi. Furthermore, these results suggest that kiwi are the first avian species to be described with biochemical (low affinity, high capacity CBG) and endocrine (high levels of corticosterone) characteristics similar to those of glucocorticoid resistant mammals.

4.2 Introduction

The circulating concentration of corticosterone in plasma is dependent on its rate of secretion, uptake by tissues and metabolic clearance by the kidneys and liver (Siiteri et al., 1982; Wingfield et al., 1984; Hammond, 1995). However, the rate of uptake by tissues and metabolic clearance can be significantly decreased by corticosterone binding to corticosteroid binding globulin (CBG) (Westphal, 1971; Hammond, 1995). Approximately 80 – 90% of corticosteroids circulate bound in dynamic equilibrium with CBG (Westphal, 1969). Therefore CBG plays an important role in determining the bio-availability of corticosterone (Siegel, 1995).

Corticosteroid binding globulin has been identified in virtually every vertebrate species studied to date, including at least 30 avian species (Seal and Doe, 1963; Westphal, 1971; Wingfield et al., 1984; Hammond, 1995; Carsia and Harvey, 2000). Circulating concentrations of CBG have been shown to vary with sex (Siiteri et al., 1982; Silverin, 1986; Bradley and Stoddart, 1992), season (Silverin, 1986; Klukowski et al., 1997; Romero and Wingfield, 1998; Romero et al., 1998) and with time of day (Siegel et al., 1976; Kovacs and Peczely, 1983). However, significant changes in plasma CBG concentrations are unlikely to occur over short periods of about an hour (Siiteri et al., 1982).

Avian CBG has been characterised as a monomeric glycoprotein with a molecular weight of approximately 55 – 63 kDa (Gould and Siegel, 1978b). It is a highly specific protein, binding corticosteroids and progesterone with very high affinity (10^{-8} – 10^{-10} M) and low capacity (10^{-8} – 10^{-9} M) (Clark and Peck Jr, 1977; Wingfield et al., 1984). The binding of corticosterone to CBG is primarily due to hydrogen, ionic and hydrophobic bonds (Westphal, 1971). CBG is primarily produced by the liver in birds (Siiteri et al., 1982). Although largely unexplored in avian species (Carsia and Harvey, 2000), extrahepatic synthesis of CBG is becoming increasingly apparent in mammals (Hammond, 1995). Corticosterone also circulates bound to plasma albumins of weak affinity (10^{-3} – 10^{-5} M) and very high capacity (Clark and Peck Jr, 1977). However, corticosteroids bound in this manner are considered essentially free, due to the very low binding affinity of plasma albumins.

It is generally excepted that only the free, or unbound (to CBG), corticosterone is biologically active (Thompson and Lippman, 1974; Wingfield et al., 1984; Barsano and Baumann, 1989; Mendel, 1989). There is, however, mounting evidence in mammalian species that bound corticosteroids may also impart a biological influence on target tissues (Hammond, 1995). For example, bound corticosteroids can interact with plasma membranes, selectively enter target glucocorticoid cells, and also interact with elastase on the surface of neutrophils to promote delivery of glucocorticoids to target cells (Siiteri et al., 1982; Hammond et al., 1991; Hammond, 1995). Binding proteins may therefore control steroid bio-availability in a highly selective and targeted fashion (Hammond, 1995).

Measuring corticosterone concentrations using radioimmunoassay cannot distinguish between corticosterone bound to CBG and that which is free. Furthermore, apparent increases in basal levels of corticosterone may not be due to an increase in secretion, but rather due to an increase in either the binding affinity (K_d) or maximum binding capacity (B_{max}) of CBG causeing decreased clearance by the liver and kidney (Wingfield et al., 1984; Hammond, 1995). Determination of K_d and B_{max} of CBG in the plasma of avian species is therefore necessary to correctly interpret the biological significance of changes in plasma corticosterone concentrations.

This study had two objectives. Firstly, to determine the optimal conditions for the measurement of CBG in Japanese quail (*Coturnix coturnix japonica*) and kiwi (*Apteryx mantelli*) plasma. Secondly, to determine the K_d and B_{max} of CBG in nocturnal house, outdoor pen and wild kiwi. This would allow for a more precise interpretation of the unusually high levels of corticosterone found in kiwi plasma (chapter 3).

4.3 Materials and methods

4.3.1 General procedure for the preparation of steroid free plasma

Equal volumes of plasma and dextran-coated charcoal (DCC; 12.5 g/l charcoal, 1.25 g/l dextran in PBSG) were mixed and vortexed at 5 min intervals for 30 min at 4°C. This solution was then left to stand for 90 min at 4°C, before being centrifuged at 3 000 rpm for 15 min (4°C, Heraeus Christ 5000S refrigerated centrifuge). A further spin at 13 000 rpm for 5 min was required to remove all DCC from solution. The supernatant was poured off and diluted to 2% in PBSG, forming the receptor solution used to determine K_d and B_{max}. This general procedure was used in experiments 1, 2, 3 and 4. A modified version of this general procedure was also used in experiments 4 and 5. These modifications are outlined in Table 4.3.2 (p 84) and in sections 4.3.6 and 4.3.7.

Due to the limited amount of plasma available, it was necessary to pool the plasma from several individuals to form the steroid free plasma. The numbers of individuals used in each pool are outlined in each experiment (see sections 4.3.3 to 4.3.7).

4.3.2 General procedure for the determination of K_d and B_{max} for CBG

The following general protocol was used in all experiments with only the concentrations of corticosterone standard (Sigma) and hot ligand (^{3}H-corticosterone, Amersham, UK) varying between experiments. The concentrations used are outlined in each experiment (sections 4.3.3 to 4.3.7).

Duplicate 500 µl aliquots of receptor solution were incubated with decreasing amounts of corticosterone standard and a constant amount of hot ligand. This solution was vortexed gently and left to incubate overnight at 4°C. Steroid bound in the plasma was separated from unbound steroid by addition of 500 µl of dextran-coated charcoal (2.5 g/l charcoal, 0.25 g/l dextran in PBSG) for 15 minutes at 4°C. Tubes were then centrifuged at 3 000 rpm for 15 min at 4°C and the supernatant poured off. A 3 ml

aliquot of scintillant (5 g/l PPO, 0.3 g/l dimethyl POPOP in toluene) was then added to the supernatant. The supernatant/scintillant mix was shaken for 1 h (Chiltern Scientific SS70 orbital shaker), and then left for 1 h at room temperature. Each sample was then placed on a liquid scintillation counter (Wallac 1409-411), and counted for 5 min.

4.3.3 Experiment 1. Verifying methodology using pooled quail plasma

A pool of plasma was formed using four short day (8L:16D) male quails. This pool was then assayed three times. The general procedure outlined in section 4.3.1 was used to remove the endogenous steroids from plasma. The following modifications to the general procedure outlined in section 4.3.2 were used to determine K_d and B_{max}. A serial dilution of corticosterone standard (100 → 0.78 ng/ml) was added to solution with a constant amount of hot ligand (10 000 cpm).

4.3.4 Experiment 2. Testing of methodology using pools of kiwi plasma

Plasma from five individual kiwi from each group (nocturnal house, outdoor pen and wild, see chapter 1 for group definitions) were used to form plasma pools. Each pool was analysed twice. The general procedure outlined in section 4.3.1 was used to remove the endogenous steroids from plasma. The following modifications to the general procedure (outlined in section 4.3.2) were used to determine K_d and B_{max}. A serial dilution of corticosterone standard (100 → 0.78 ng/ml) was added to solution with a constant amount of hot ligand (10 000 cpm).

4.3.5 Experiment 3. The effect of varying amounts and concentrations of hot and cold competing ligand

Plasma from five kiwi and five quail were used to form the plasma pools used in this experiment. The general procedure outlined in section 4.3.1 was used to remove the endogenous steroid from plasma. Varying amounts of corticosterone standard and label

(as outlined in Table 4.3.1) were competed against each other to determine the optimal concentrations for the measurement of K_d and B_{max} in quail and kiwi plasma.

Table 4.3.1 Concentrations of standard and amount of hot ligand used to determine optimal concentrations for the measurement of K_d and B_{max} in quail and kiwi plasma.

Species	Concentration of the top corticosterone standard (ng/ml)	Amount of hot ligand (cpm)
Kiwi	1000	10 000
Kiwi	1000	5 000
Kiwi	100	2 500
Quail	1000	10 000
Quail	1000	5 000
Quail	1000	2 500
Quail	100	2 500

Note: A single pool for each species was used in this experiment.

4.3.6 Experiment 4. The effect of plasma stripping method on corticosterone concentration, K_d and B_{max} of kiwi and quail plasma

Plasma from four kiwi and four quail were used to form the plasma pools used in this experiment. To prepare the steroid free plasma, the general protocol outlined in section 4.3.1 was modified according to the conditions outlined in Table 4.3.2. Determination of K_d and B_{max} in kiwi plasma was achieved by competing a serial dilution of corticosterone standard (1000 → 7.8 ng/ml) with a constant amount of hot ligand (10 000 cpm). Determination of K_d and B_{max} in quail plasma was achieved by competing a serial dilution of corticosterone standard (100 → 0.78 ng/ml) with a constant amount of hot ligand (10 000 cpm). The concentration of corticosterone in the plasma before the removal of endogenous steroid was determined previously in chapter 3. The corticosterone concentration of stripped plasma was determined by E.J. Candy and E. Bennett, using the same protocol outlined in chapter 3.

Table 4.3.2 Plasma stripping conditions tested in this experiment for each species.

Species	DCC addition method	Temperature
Kiwi	DCC+B	20°C
Kiwi	DCC+B	36°C
Quail	DCC+B	4°C
Quail	DCC+B	20°C
Quail	DCC+B	36°C
Quail	DCC/P	4°C
Quail	DCC/P	36°C

Note: DCC+B= DCC in buffer added in equal volumes to plasma. DCC/P= DCC added directly to the plasma. All groups were shaken at 5 min intervals for 30 min and left to stand for 90 min at their respective temperatures.

4.3.7 Experiment 5. Measurement of K_d and B_{max} in nocturnal house, outdoor pen, wild and regularly handled kiwi

Plasma from three individual kiwi were used to form each pool of plasma. For each group (nocturnal house, outdoor pen and wild, refer to p 10), three pools of plasma were created. In regularly handled kiwi (refer to p 10), a single pool of plasma was formed using the plasma from three individuals.

The following modifications were made to the general protocol outlined in section 4.3.1. Each plasma pool was stripped of endogenous steroid by mixing equal amounts of DCC/buffer solution and plasma at 36°C. This was then shaken at 5 min intervals for 30 min and then left to stand for a further 30 min (at 36°C) before separation and dilution. The following modifications to the general procedure outlined in section 4.3.2 were used to determine K_d and B_{max} in kiwi. A serial dilution of corticosterone standard (1000 → 7.8 ng/ml) was competed with a constant amount of hot ligand (10 000 cpm).

4.3.8 Statistical analysis

Since the total mass of steroid hormone added is known, the bound/unbound (B/F) ratio can be used to calculate the total mass of hormone bound (Scatchard, 1949). Total

binding was corrected for non-specific binding as suggested by Chamness and McGuire (1975). The slope of the line fitted to the specific binding curve (by linear regression) is the association constant (K_a), and its reciprocal is the dissociation constant (K_d). All binding affinities are expressed as nmol/litre (nM). The intersection of the line with the abscissa is the binding capacity in nmol/litre (nM). Correction for non-specific binding (i.e. transformation of total binding curve to specific binding) will distort the experimental error. Furthermore, in Scatchard analysis the value of Y (the B/F ratio) is used to calculate the value of X (bound hormone). This violates an assumption of linear regression that all uncertainty is in the Y axis while X is known precisely. This method is therefore unlikely to provide an accurate determination of K_d and B_{max}.

Analysis of such data using non-linear regression will determine K_d and B_{max} more accurately. The non-linear regression equation used for one site binding systems is shown below. It describes the binding of a ligand to a receptor that follows the law of mass action.

Equation 1 This equation describes the equilibrium binding of a ligand to a receptor as a function of increasing ligand concentration

$$y = \frac{B_{max} * X}{(K_d + X)}$$

X = concentration of ligand

y = specific binding of the ligand

B_{max} = maximum binding capacity of CBG

K_d = equilibrium dissociation constant

Analysis for homogeneity of variance between groups was performed using a Bartletts test (SYSTAT version 5.02, Systat Inc, Illinois). Two-way single measures ANOVA with post hoc linear contrasts, and two sample t-tests were performed where appropriate using SYSTAT version 8.0 (SPSS, Inc). Linear and non-linear regression analyses were performed using GraphPad Prism version 3.02 (1999; GraphPad Software Inc.).

4.4 Results

4.4.1 Experiment 1. Verifying methodology using pooled quail plasma

4.4.1.1 Scatchard analysis

In quail, total binding for each replicate demonstrated clear regions of specific (S) and non-specific (NS) binding (Figure 4.4.1, p 91). The presence of a straight horizontal line for NS binding confirms the presence of a low affinity and high capacity NS binding system relative to the receptor system. Calculation of K_d and B_{max} demonstrate the presence of a high affinity (~10^{-8} M) and low capacity (~10^{-8} M) binding site (Figure 4.4.1. p 91). The coefficients of variation (CV) for K_d and B_{max} were well below 10%. Mean, S.E.M. and percent CV for K_d and B_{max} are summarised in Table 4.4.3 (p 115).

4.4.1.2 Non-linear regression analysis

Non-specific binding increased linearly with increasing concentrations of cold ligand (Figure 4.4.2, p 92). Conversely, specific binding reached a theoretical maximum (B_{max}). This analysis also demonstrates that cold ligand will preferentially bind to the high affinity S binding sites (Figure 4.4.2, p 92).

The K_d calculated using Scatchard analysis was approximately 20 fold higher than that calculated with non-linear regression (t=23.38, df=2, *P*=0.002, Table 4.4.3, p 115). Scatchard analysis and non-linear regression analysis did not differ in their calculation of B_{max} (t=0.122, df=4, *P*=0.909, Table 4.4.3, p 115).

4.4.2 Experiment 2. Testing of methodology using pools of kiwi plasma

4.4.2.1 Scatchard analysis

All groups (nocturnal house, outdoor pen, wild) and replicates did not show clear S and NS binding regions of the total binding curve (Figure 4.4.3, p 93). Therefore, K_d and B_{max} for S binding could not be calculated.

4.4.2.2 Non-linear regression analysis

For all groups and replicates, S and NS binding increased at similar rates (Figure 4.4.4, p 94).

4.4.3 Experiment 3. The effect of varying amounts and concentrations of hot and cold competing ligand

4.4.3.1 Scatchard analysis

In kiwi plasma, the increased concentration of competing cold ligand (1 000 ng/ml top standard) resulted in distinct S and NS binding regions of the total binding curve (top panel, Figure 4.4.5, p 95). The presence of a straight horizontal line for NS binding confirms the presence of a low affinity and high capacity NS binding system relative to the receptor system. Calculation of K_d and B_{max} demonstrate the presence of a medium-high affinity (~10^{-6} M) and medium-low capacity (~10^{-6} M) binding site (relative to NS binding). Competing 1000 ng/ml cold ligand with a decreased amount of hot ligand (5 000 cpm) produced similar results (middle panel, Figure 4.4.5, p 96.). Total binding was not saturated when the amounts of competing cold (100 ng/ml) and hot (2 500 cpm) ligand were reduced (bottom panel, Figure 4.4.5, p 95).

In quail plasma, competing high amounts of hot (10 000 cpm) and cold (1000 ng/ml) ligand resulted in distinct S and NS binding regions of the total binding curve (top left panel, Figure 4.4.6, p 96). In addition, the B/F ratio actually started to increase at high concentrations of bound cold ligand (top left panel, Figure 4.4.6, p 96). A similar trend was also demonstrated when the amount of hot ligand was reduced to 5 000 cpm or 2 500 cpm (top right and bottom left panels, Figure 4.4.6, p 96). However, decreasing the amount of cold competitor to 100 ng/ml resulted in distinct S and NS binding regions of the total binding curve, without the increase in B/F ratios as seen previously (bottom right panel, Figure 4.4.6, p 96).

4.4.3.2 Non-linear regression analysis

In kiwi, non-specific binding increased linearly with increasing concentrations of cold ligand (top panel, Figure 4.4.7, p 97). Conversely, specific binding reached a theoretical maximum (B_{max}). This analysis also demonstrates that cold ligand will preferentially bind to the high affinity S binding sites in kiwi plasma (top panel, Figure 4.4.7, p 97). Decreasing the amount of competing hot ligand to 5 000 cpm appears to have little influence on S and NS binding (middle panel, Figure 4.4.7, p 97). However, by decreasing the amount of competing hot and cold ligand to 2 500 cpm and 100 ng/ml respectively, S and NS binding increased at similar rates (bottom panel, Figure 4.4.7, p 97).

In quail, non-linear regression analysis revealed that NS binding increases at a greater rate than S binding (top left panel, Figure 4.4.8, p 98). This trend was consistent for all situations where a high concentration of cold ligand (1000 ng/ml) was used, regardless of the amount of competing hot ligand (top right and bottom left panels, Figure 4.4.8, p 98). However, decreasing the concentration of cold competitor to 100 ng/ml resulted in distinct S and NS binding (bottom right panel, Figure 4.4.8, p 98).

4.4.4 Experiment 4. The effect of plasma stripping method on corticosterone concentration, Kd and Bmax of kiwi and quail plasma

4.4.4.1 Scatchard analysis:

Increasing the temperature during stripping had no effect on the values of K_d and B_{max} of kiwi CBG (Figure 4.4.9, p 99). However, it did result in an increase in the percentage of endogenous corticosterone stripped from the plasma (~60% at 4°C, ~75% at 20°C and ~93% at 36°C, Table 4.4.1, p 105).

Increasing the temperature during stripping from 4°C to 20°C had little effect on K_d and B_{max} in quail (top and middle panels, Figure 4.4.10, p 100). However, there was a lack of distinct S and NS binding regions of the total binding curve when the temperature is increased to 36°C. Increases in temperature had little effect on the amount of endogenous corticosterone stripped from quail plasma (Table 4.4.2, p 106).

Adding DCC directly to quail plasma appears to have little influence the amount of endogenous corticosterone stripped from the plasma, however small sample size precluded statistical analysis (Table 4.4.2, p 106). This method of stripping appears not to affect the K_d and B_{max} of quail CBG (top panel, Figure 4.4.11, p 101 compared with Figure 4.4.10, p 100). However, there is also a lack of distinct S and NS binding regions of the total binding curve when the temperature is increased to 36°C for this DCC addition method (Figure 4.4.11, p 101).

4.4.4.2 Non-linear regression analysis:

Increasing the temperature to 36°C during stripping of quail plasma results in a significant decrease in S binding (Figure 4.4.12, p 102). Furthermore, total binding is almost totally due to NS binding (Figure 4.4.12, p 102). Adding DCC directly to quail plasma and stripping at 36°C reveals an identical trend (Figure 4.4.14, p 104).

Increasing the temperature appears to have had little effect on the K_d and B_{max} of binding in kiwi plasma (Figure 4.4.13, p 103).

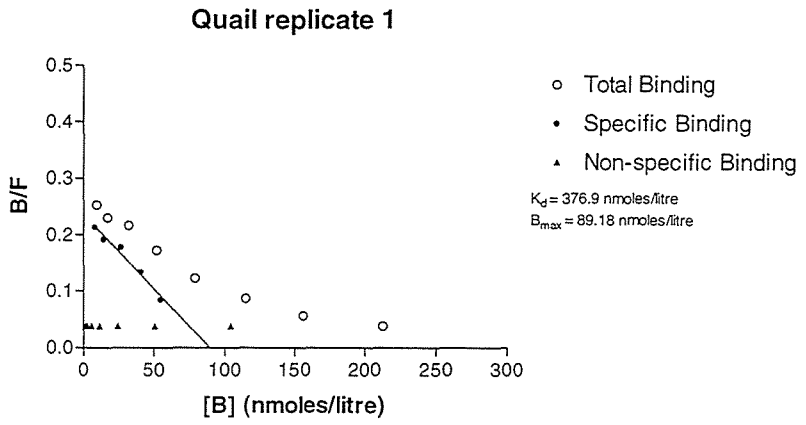
4.4.5 Experiment 5. Measurement of Kd and Bmax in nocturnal house, outdoor pen, wild and regularly handled kiwi

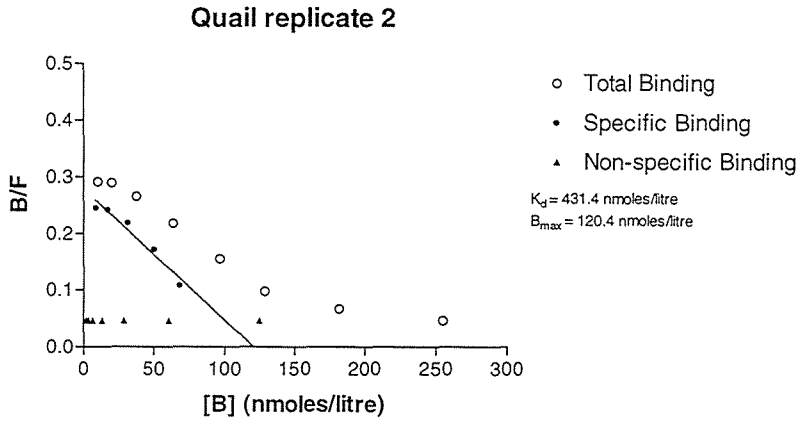
4.4.5.1 Comparison of Kd and Bmax between groups

There was no difference between Scatchard analysis and non-linear regression analysis in determining B_{max} (two-way single measures ANOVA, $F_{1,12}=3.285$, $P=0.095$, Table 4.4.3, p 115). However, there was a difference between Scatchard analysis and non-linear regression in determining K_d ($F_{1,12}=260.76$, $P<0.001$). The value of K_d determined by non-linear regression was approximately 10 fold lower than that determined by Scatchard analysis (Table 4.4.3, p 115). There was no difference in B_{max} ($F_{2,12}=1.743$, $P=0.216$) or K_d ($F_{2,12}=1.372$, $P=0.291$) between nocturnal house (Figures 4.4.15, 4.4.18, pp 107, 110), outdoor pen (Figures 4.4.16, 4.4.19, pp 108, 111) and wild kiwi (Figures 4.4.17, 4.4.20, pp 109, 112).

4.4.5.2 Comparison of corticosterone concentration and Bmax for each group

Plasma levels of corticosterone were similar to maximum binding capacity (B_{max}) in outdoor penned kiwi 30 min and 60 min after capture (Figure 4.4.21, p 113). Peak corticosterone levels did not reach the maximum binding capacity for any other group at any time (Figure 4.4.21, p 113). However, the coefficient of variation for B_{max} in outdoor penned kiwi was 14.54% (Scatchard analysis, Table 4.4.3, p 115). For all other groups, the coefficients of variation were less than 10% (Table 4.4.3, p 115). Plasma levels of corticosterone did not reach B_{max} in regularly handled kiwi before or after a period of public display (Figure 4.4.22, p 114). Small sample sizes precluded statistical analysis.





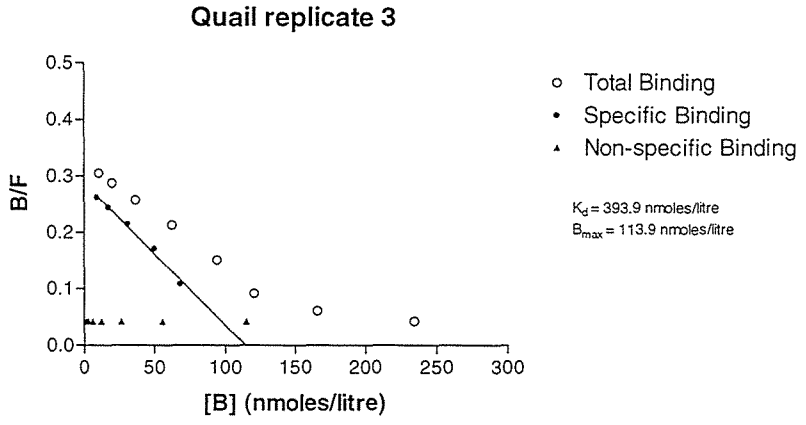


Figure 4.4.1 Scatchard plots for the binding of corticosterone to CBG for a single pool of plasma collected from short day male quail. K_d and B_{max} for each replicate are indicated in the legends.

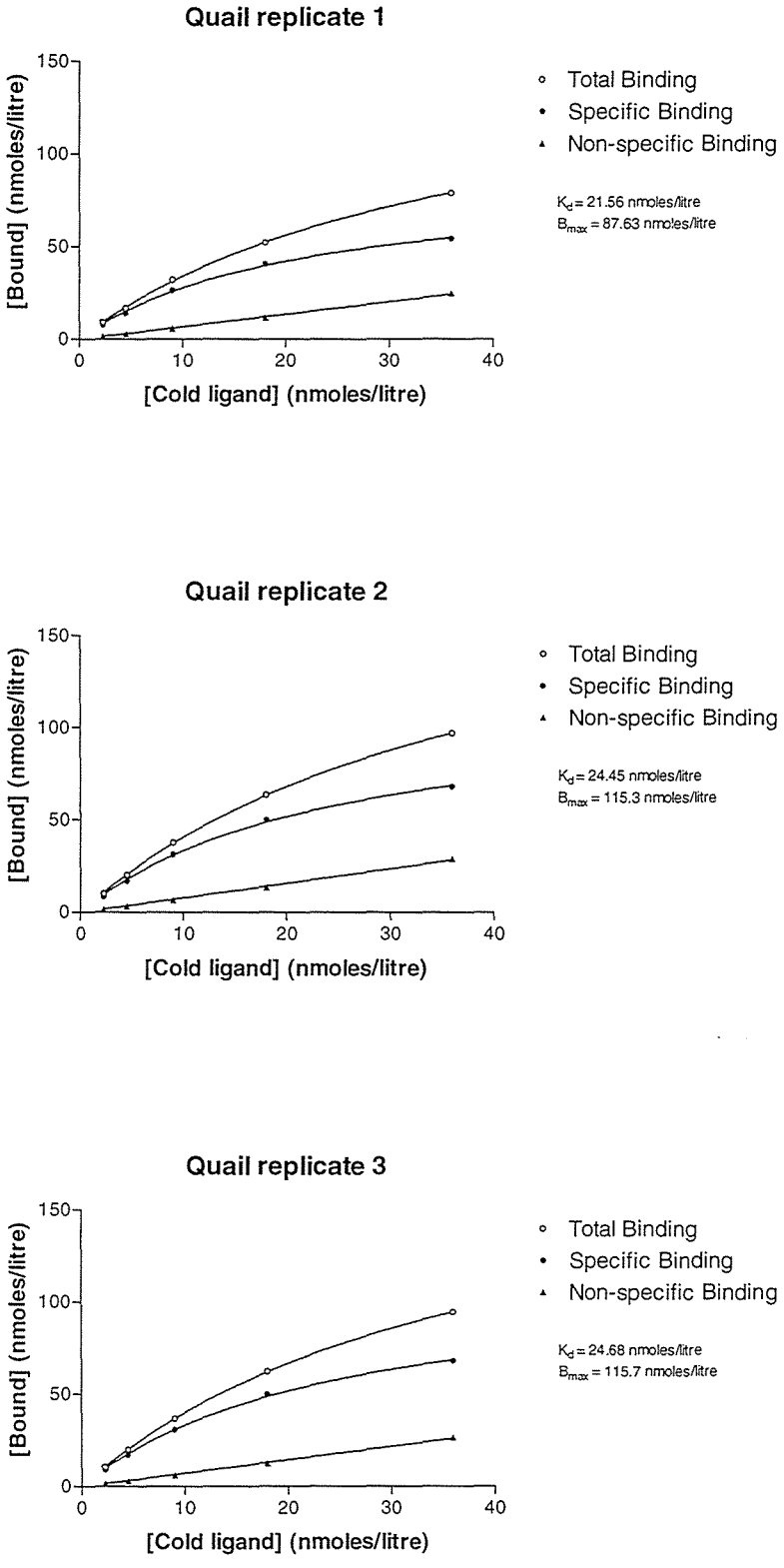


Figure 4.4.2 Non-linear regression plots for the binding of corticosterone to CBG for a single pool of plasma collected from short day male quail. K_d and B_{max} for each replicate are indicated in the legends.

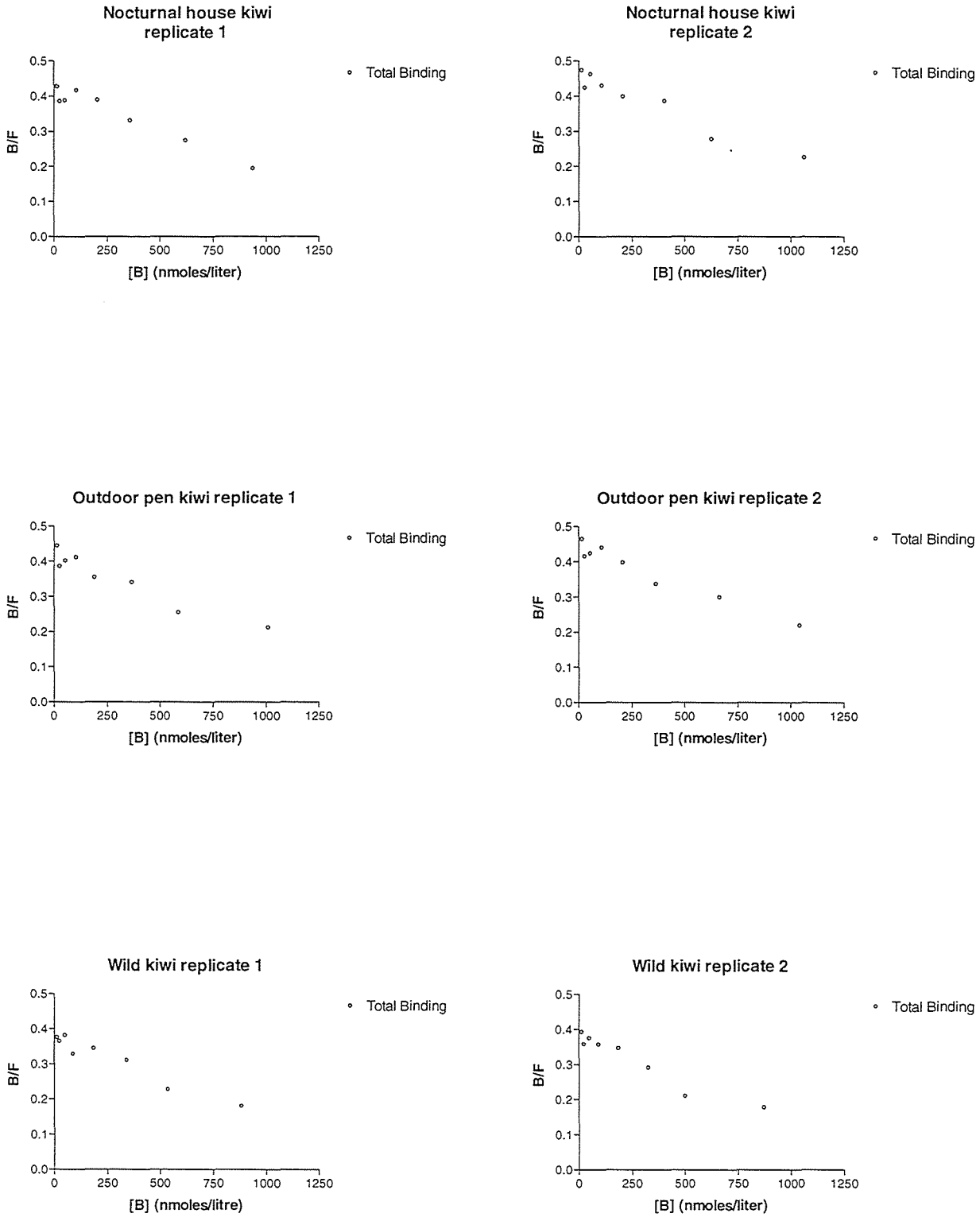


Figure 4.4.3 Scatchard plots for the binding of corticosterone to CBG in pools of plasma collected from nocturnal house, outdoor pen and wild kiwi. Each pool was analysed twice (replicates 1 and 2).

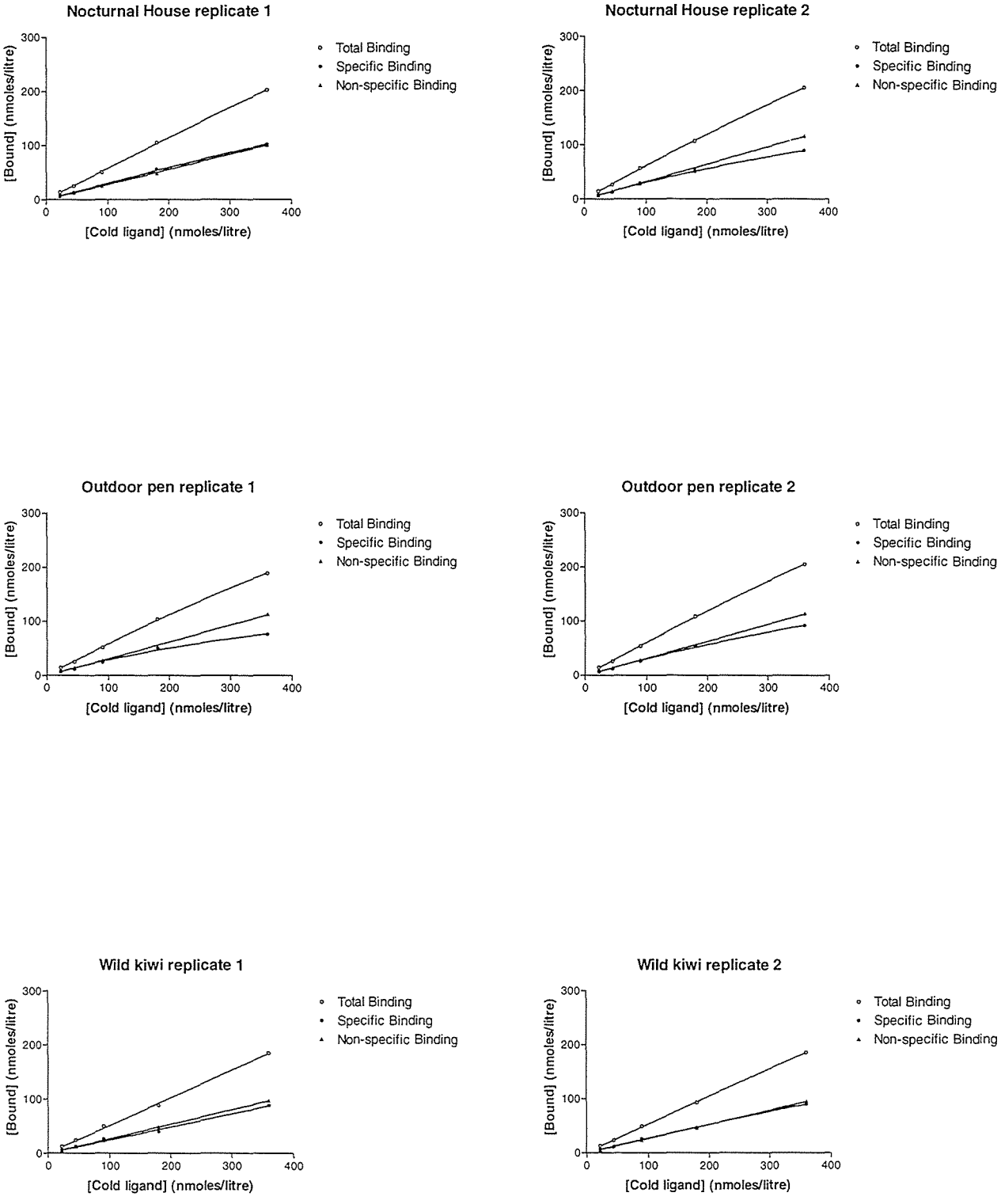


Figure 4.4.4 Non-linear regression plots for the binding of corticosterone to CBG in pools of plasma collected from nocturnal house, outdoor pen and wild kiwi. Each pool was analysed twice (replicates 1 and 2).

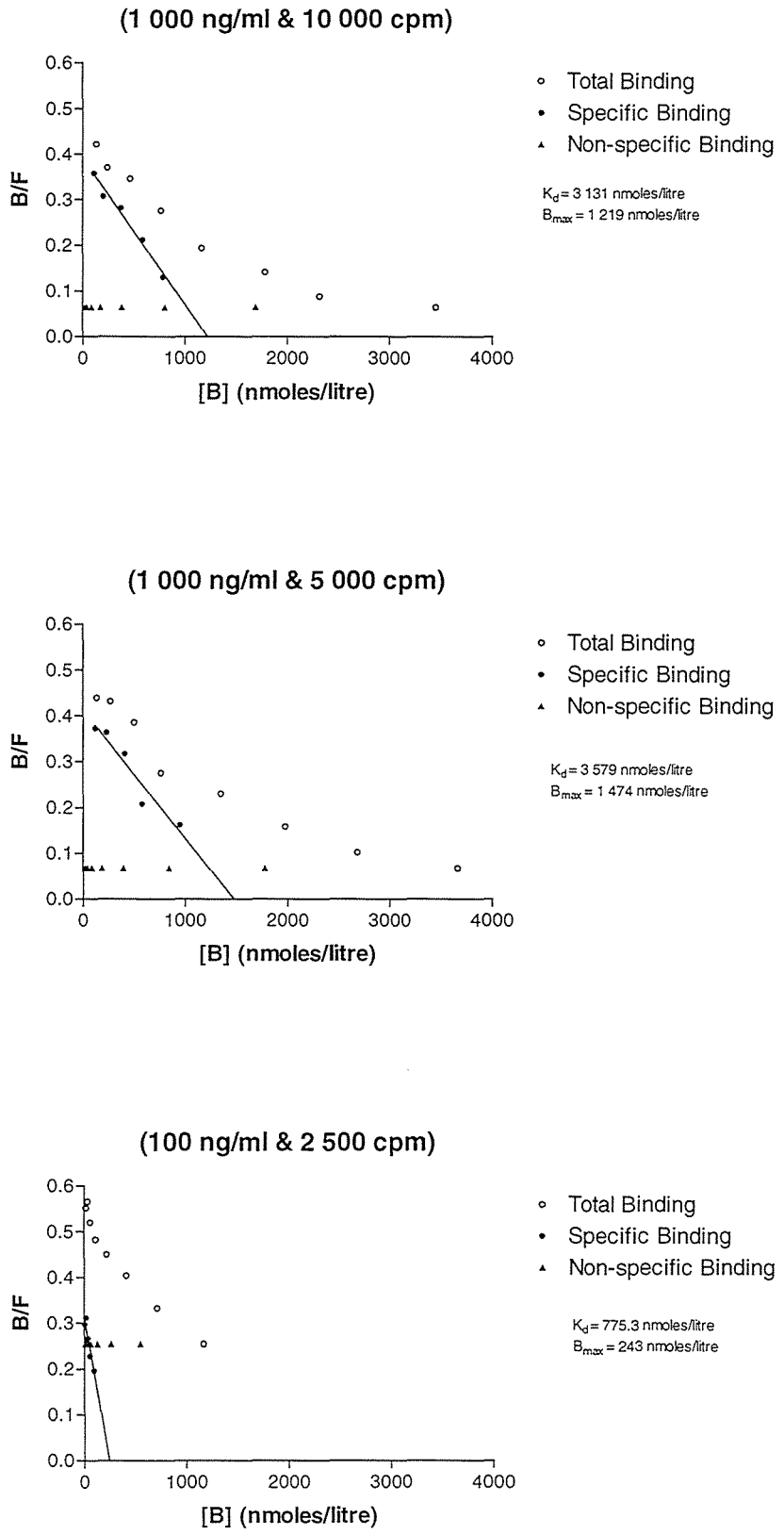


Figure 4.4.5 Scatchard plots for the binding of corticosterone to CBG for a single pool of plasma collected from kiwi. The concentrations of cold and hot ligand used in each experiment are indicated above their respective graphs. K_d and B_{max} for each experiment are indicated in the legends.

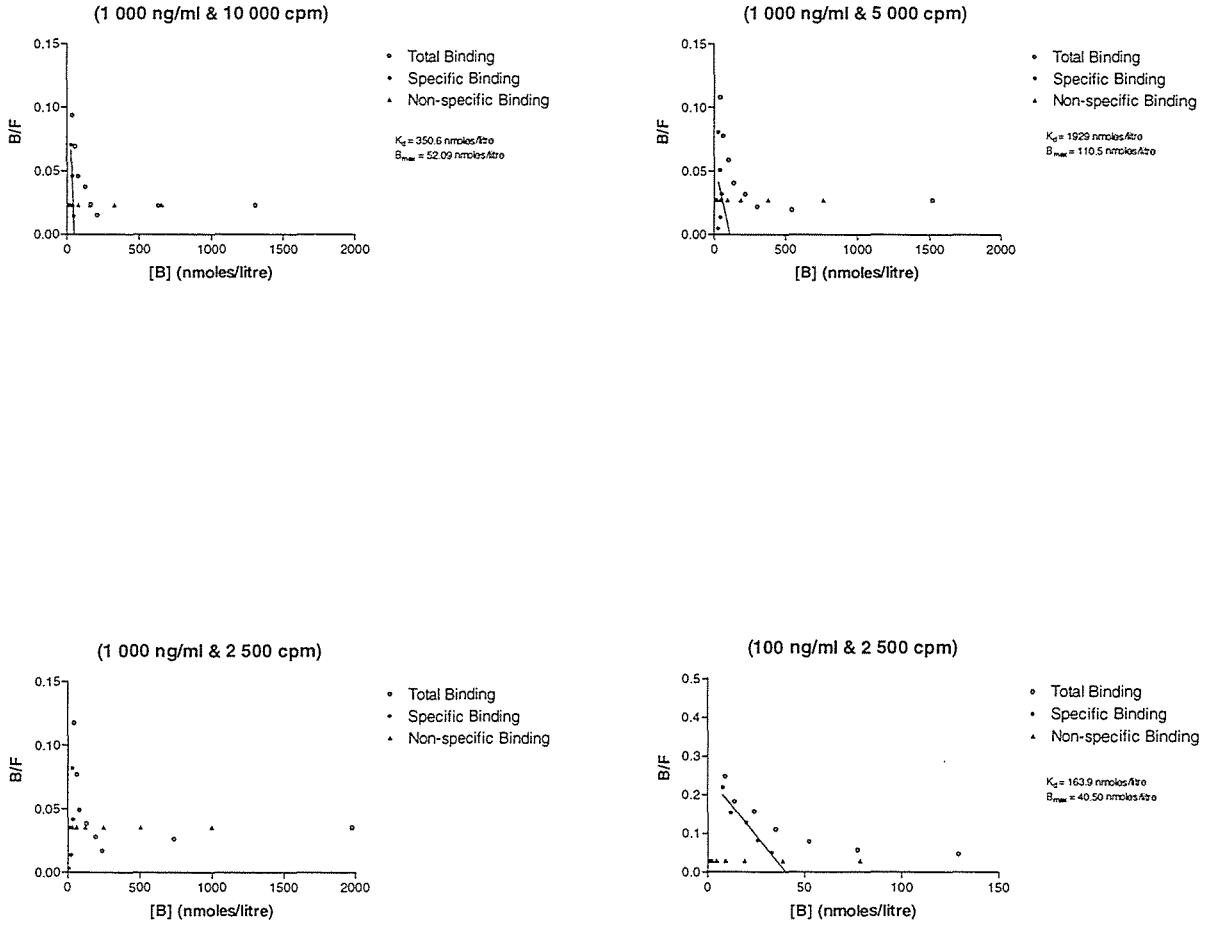


Figure 4.4.6 Scatchard plots for the binding of corticosterone to CBG for a single pool of plasma collected from quail. The concentrations of cold and hot ligand used in each experiment are indicated above their respective graphs. K_d and B_{max} for each experiment are indicated in the legends.

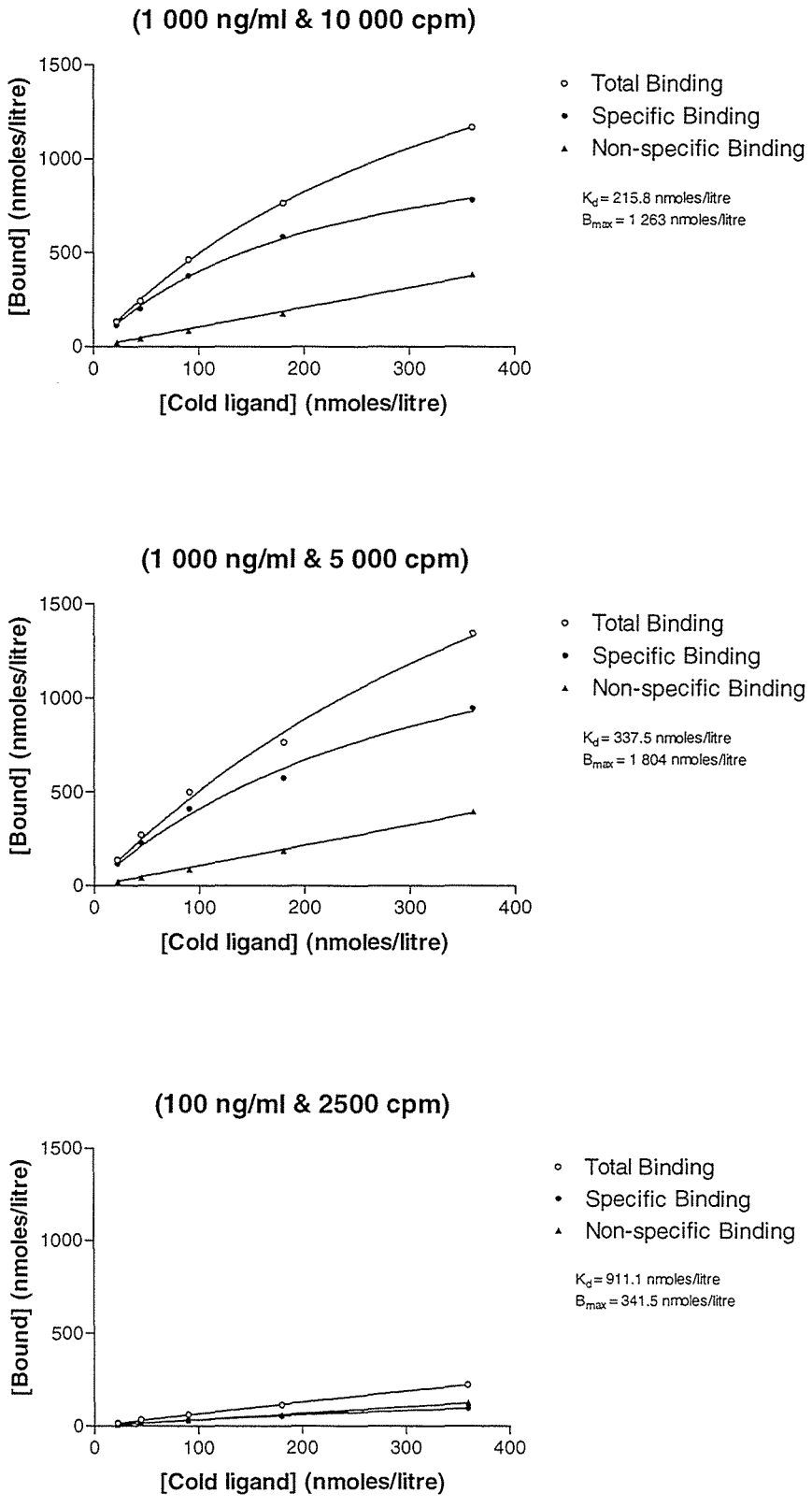


Figure 4.4.7 Non-linear regression plots for the binding of corticosterone to CBG for a single pool of plasma collected from kiwi. The concentrations of cold and hot ligand used in each experiment are indicated above their respective graphs. K_d and B_{max} for each experiment are indicated in the legends.

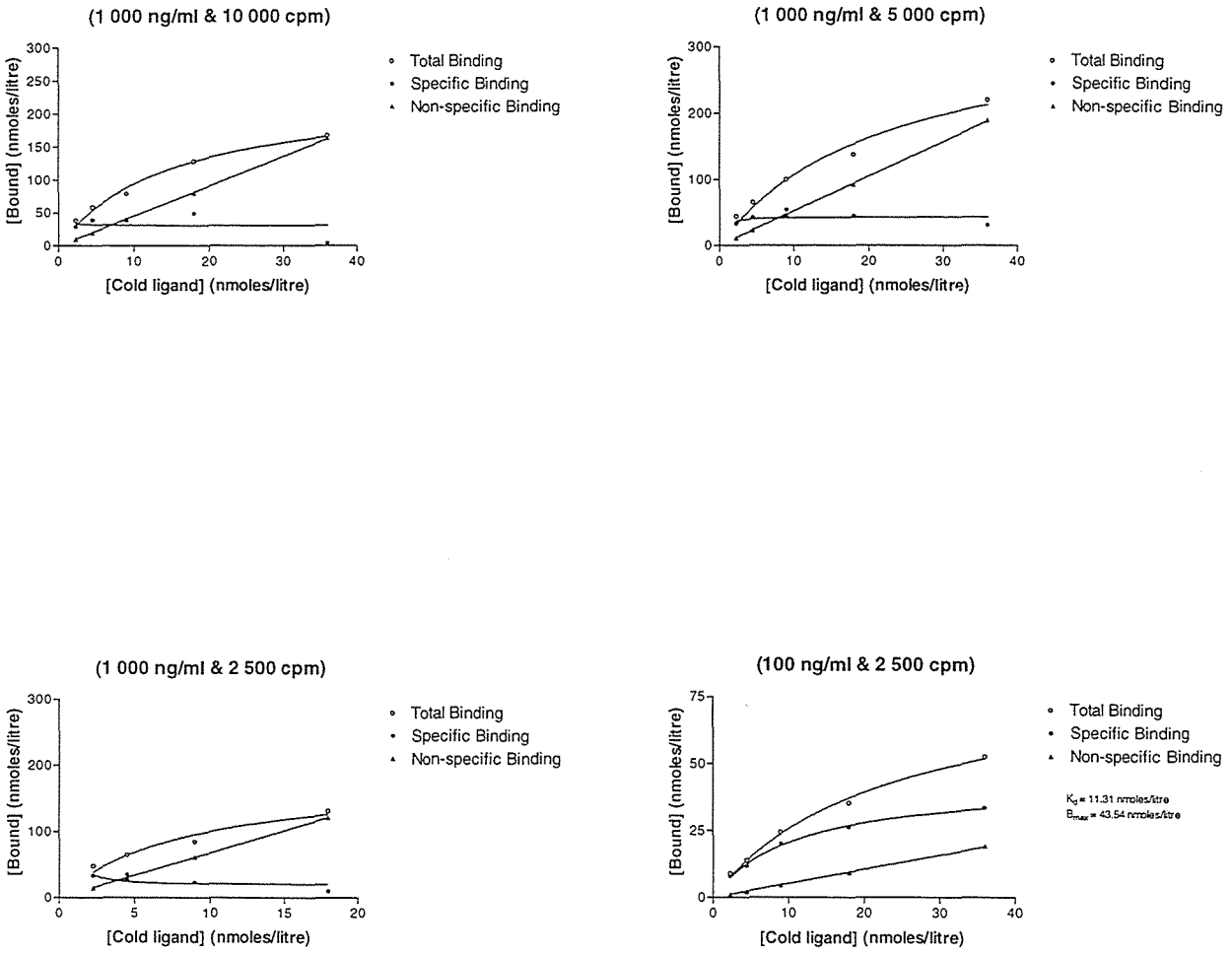
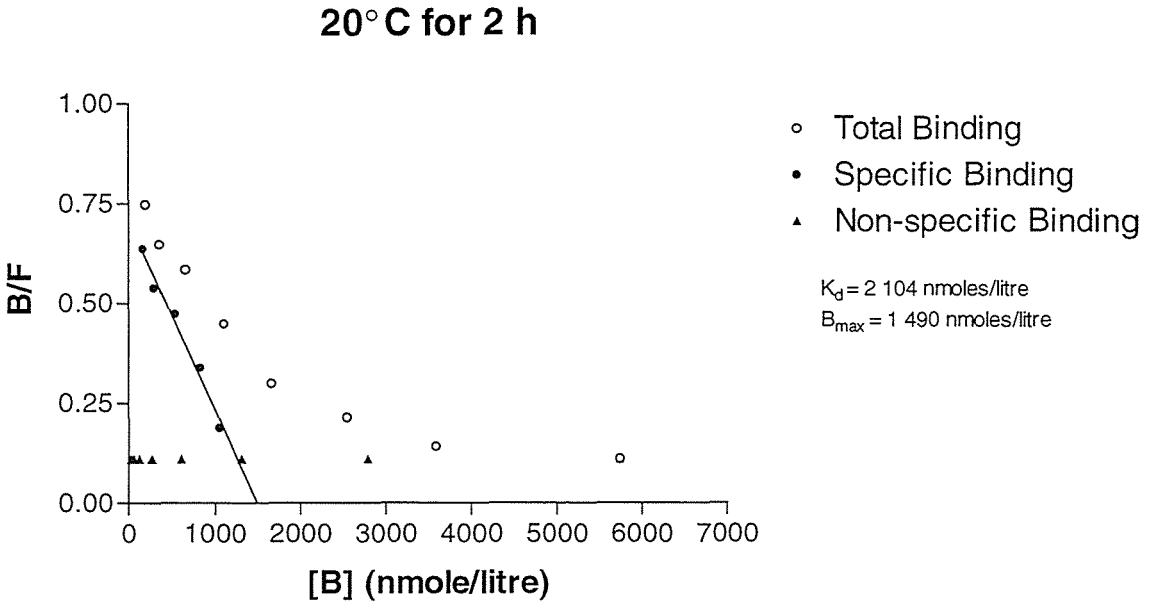


Figure 4.4.8 Non-linear regression plots for the binding of corticosterone to CBG for a single pool of plasma collected from quail. The concentrations of cold and hot ligand used in each experiment are indicated above their respective graphs. K_d and B_{max} for each experiment are indicated in the legends. Note the change in scale on the y-axis on the bottom right panel.



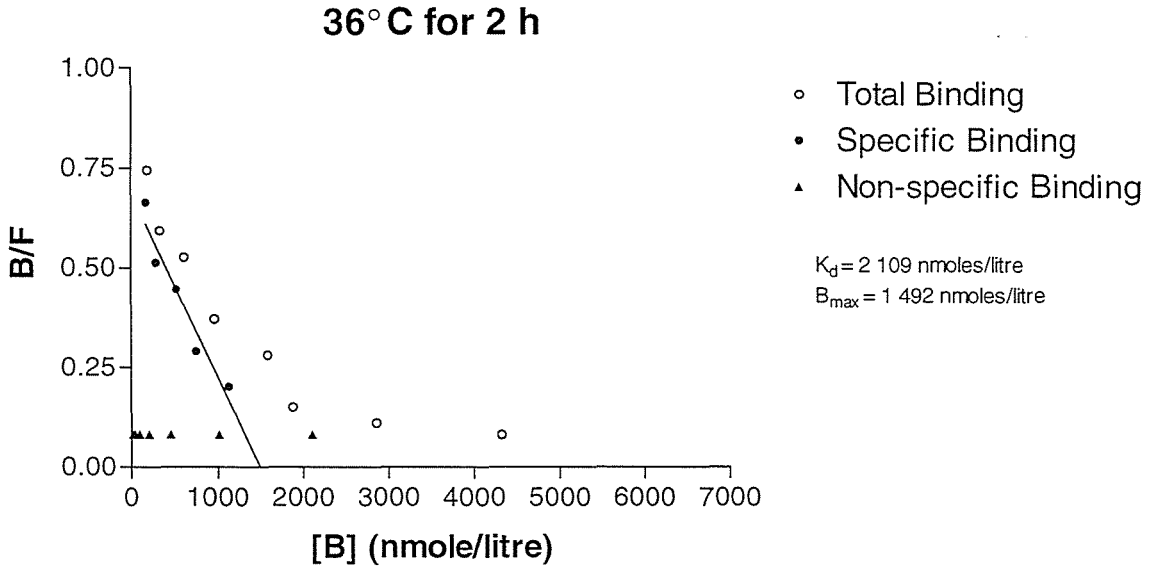


Figure 4.4.9 Scatchard plots for the binding of corticosterone to CBG for a single pool of plasma collected from kiwi. The conditions used during stripping of endogenous steroid from the plasma are indicated above their respective graphs. Also, a DCC-buffer solution was added to plasma in equal volumes. K_d and B_{max} for each experiment are indicated in the legends.

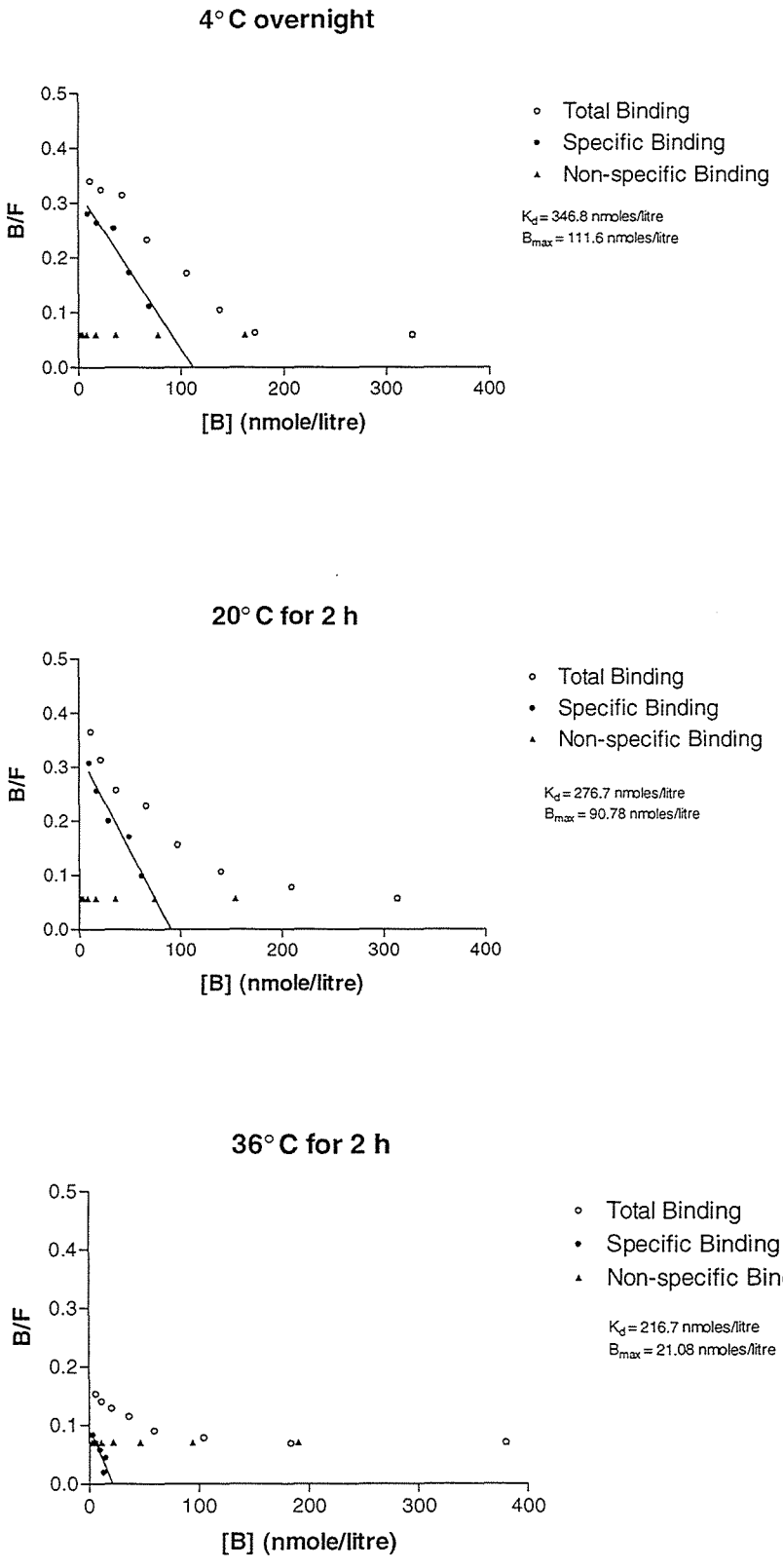
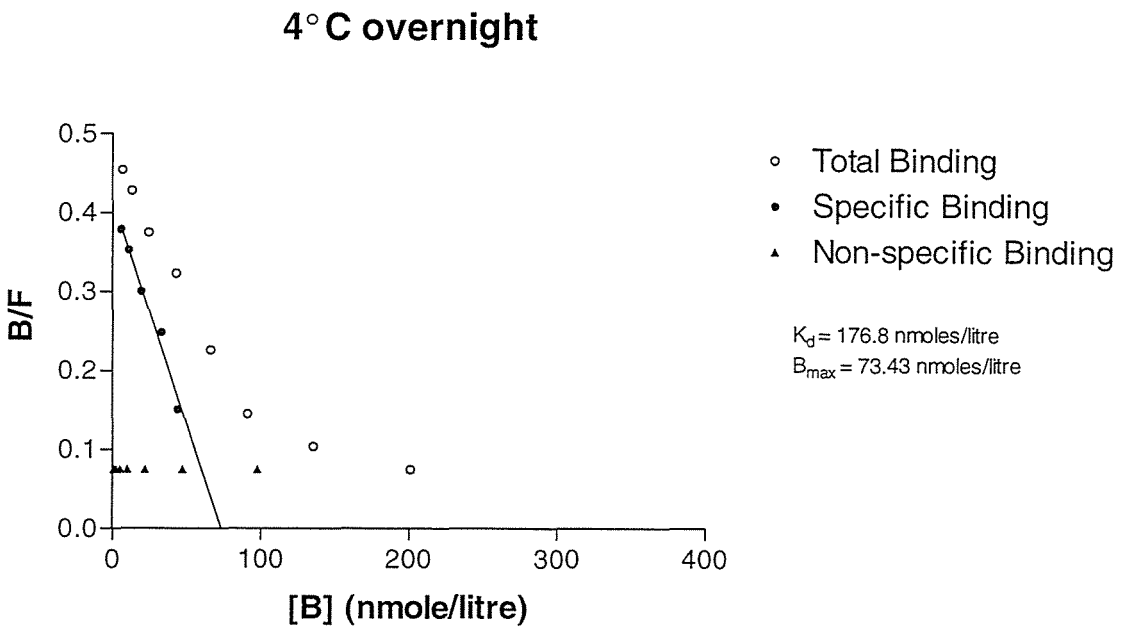


Figure 4.4.10 Scatchard plots for the binding of corticosterone to CBG for a single pool of plasma collected from quail. The conditions used during stripping of endogenous steroid from the plasma are indicated above their respective graphs. Also, a DCC-buffer solution was added to plasma in equal volumes. K_d and B_{max} for each experiment are indicated in the legends.



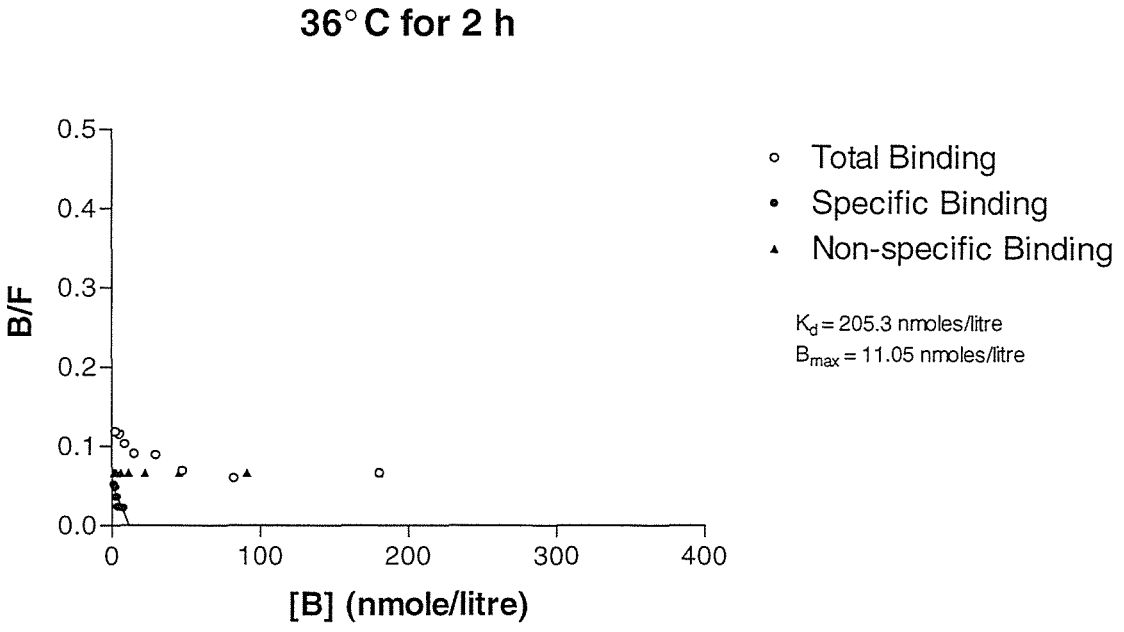


Figure 4.4.11 Scatchard plots for the binding of corticosterone to CBG for a single pool of plasma collected from quail. The conditions used during stripping of endogenous steroid from the plasma are indicated above their respective graphs. Also, DCC was added directly to the plasma. K_d and B_{max} for each experiment are indicated in the legends.

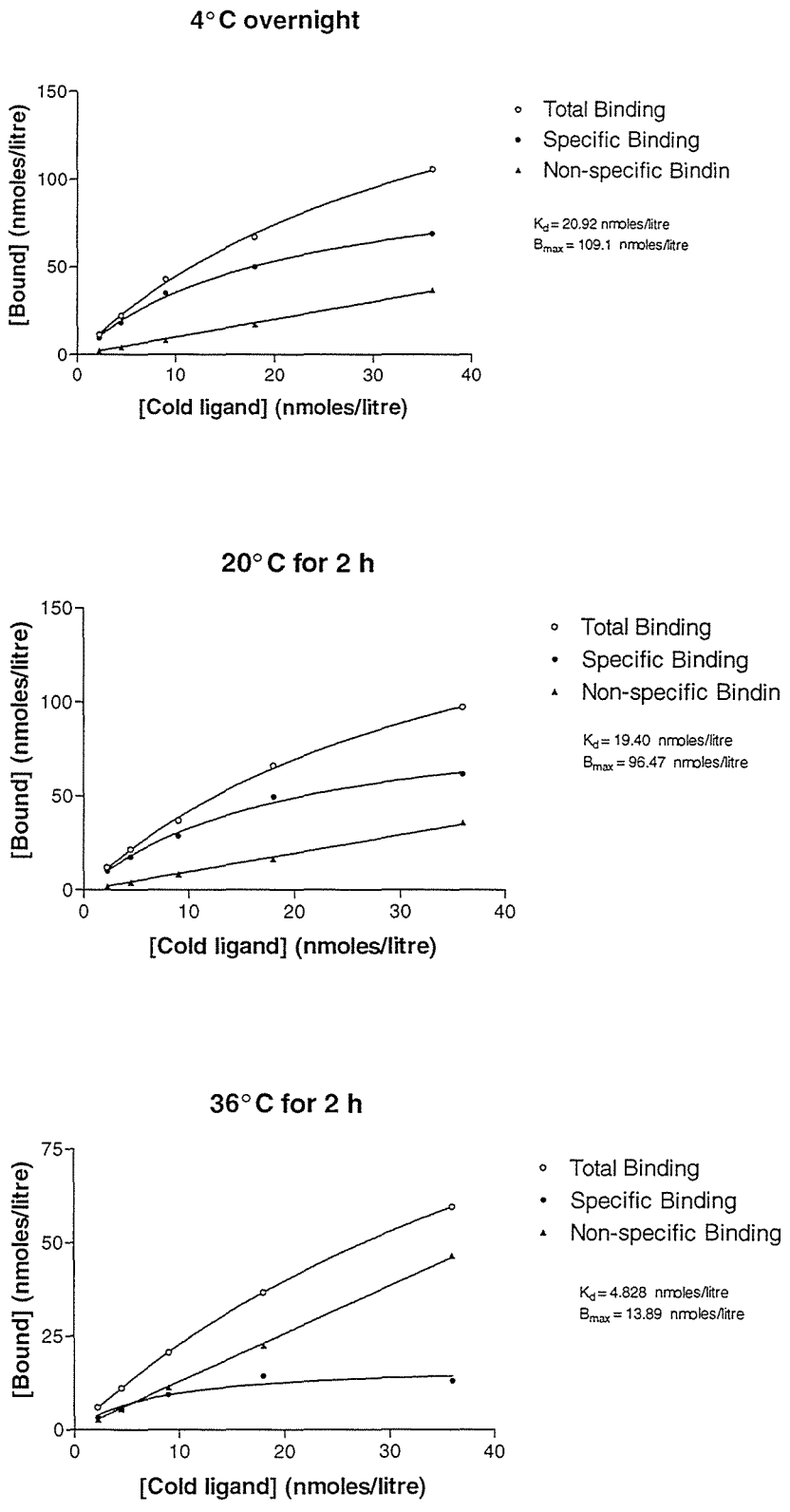
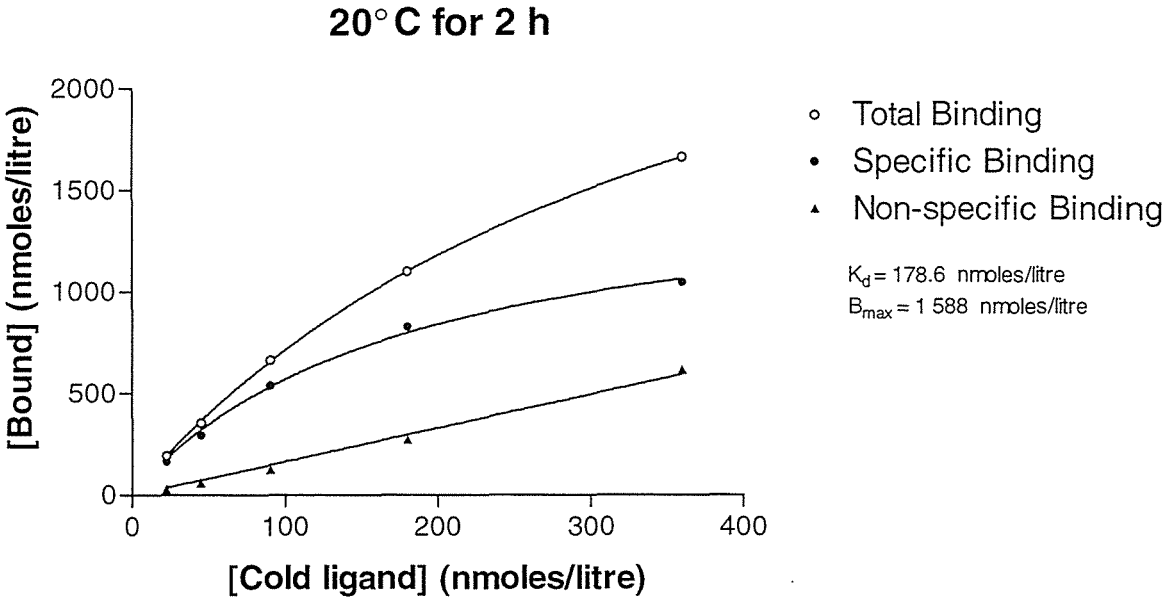


Figure 4.4.12 Non-linear regression plots for the binding of corticosterone to CBG for a single pool of plasma collected from quail. The conditions used during stripping of endogenous steroid from the plasma are indicated above their respective graphs. Also, a DCC-buffer solution was added to plasma in equal volumes. K_d and B_{max} for each experiment are indicated in the legends.



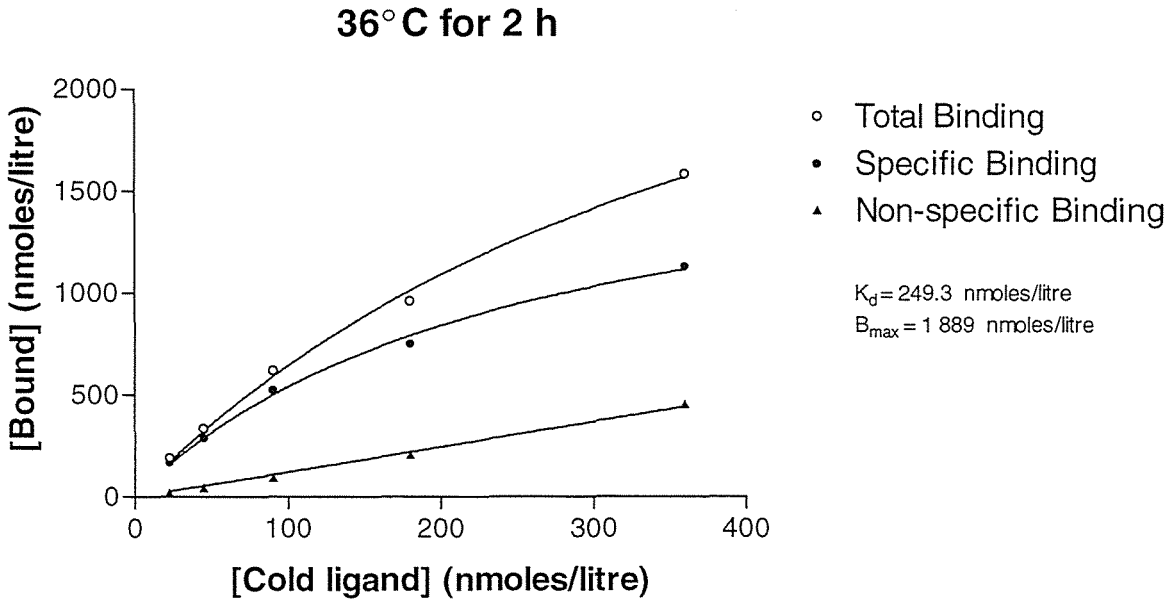


Figure 4.4.13 Non-linear regression plots for the binding of corticosterone to CBG for a single pool of plasma collected from kiwi. The conditions used during stripping of endogenous steroid from the plasma are indicated above their respective graphs. Also, a DCC-buffer solution was added to plasma in equal volumes. K_d and B_{max} for each experiment are indicated in the legends.

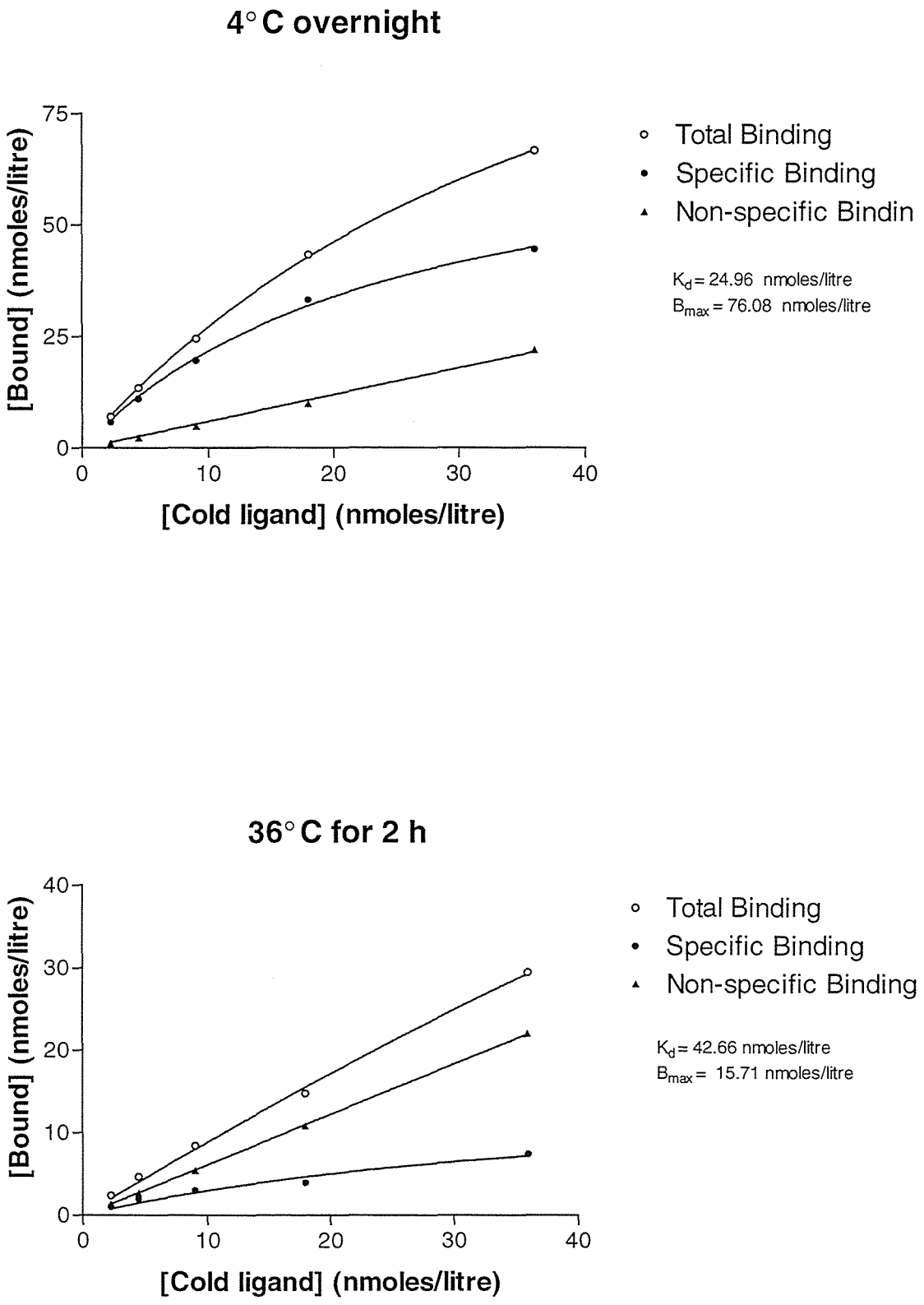


Figure 4.4.14 Non-linear regression plots for the binding of corticosterone to CBG for a single pool of plasma collected from quail. The conditions used during stripping of endogenous steroid from the plasma are indicated above their respective graphs. Also, DCC was added directly to the plasma. K_d and B_{max} for each experiment are indicated in the legends.

Table 4.4.1 Kiwi plasma concentrations of corticosterone before and after each method of removing endogenous steroid.

	Species	Treatment	Corticosterone concentration in plasma prior to stripping (ng/ml)	Corticosterone concentration in plasma after stripping (ng/ml)	Percentage of corticosterone left in plasma
Experiment 2	Kiwi	4°C overnight DCC+B	114.39 ± 39.91 (n=5)	47.26	41.31
	Kiwi	4°C overnight DCC+B	165.96 ± 89.75 (n=5)	62.24	37.50
	Kiwi	4°C overnight DCC+B	208.37 ± 61.98 (n=5)	77.91	37.39
Experiment 3	Kiwi	4°C overnight DCC+B	174.60 ± 72.80 (n=5)	70.67	40.48
Experiment 4	Kiwi	20°C for 2 h DCC+B	246.50 ± 122.57 (n=4)	65.11	26.41
	Kiwi	36°C for 2 h DCC+B	246.50 ± 122.57 (n=4)	17.13	7.19

Note: All corticosterone concentrations were determined using the methodology outline in chapter 3. DCC+B denotes the stripping method of adding equal volumes of DCC in buffer solution to plasma. Values prior to stripping are mean ± S.E.M.

Table 4.4.2 Quail plasma concentrations of corticosterone after each method of removing endogenous steroid.

	Species	Treatment	Corticosterone concentration in plasma after stripping (ng/ml)
Experiment 1	Quail	4°C overnight DCC+B	1.49
	Quail	4°C overnight DCC+B	0.90
	Quail	4°C overnight DCC+B	0.66
Experiment 3	Quail	4°C overnight DCC+B	0.79
Experiment 4	Quail	4°C overnight DCC+B	0.69
	Quail	20°C for 2 h DCC+B	0.96
	Quail	36°C for 2 h DCC+B	0.82
	Quail	20°C for 2 h DCC	2.90
	Quail	36°C for 2 h DCC	1.41

Note: All corticosterone concentrations were determined using the methodology outline in chapter 3. DCC+B denotes the stripping method of adding equal volumes of DCC in buffer solution to plasma. DCC denotes the method of adding DCC directly to the plasma.

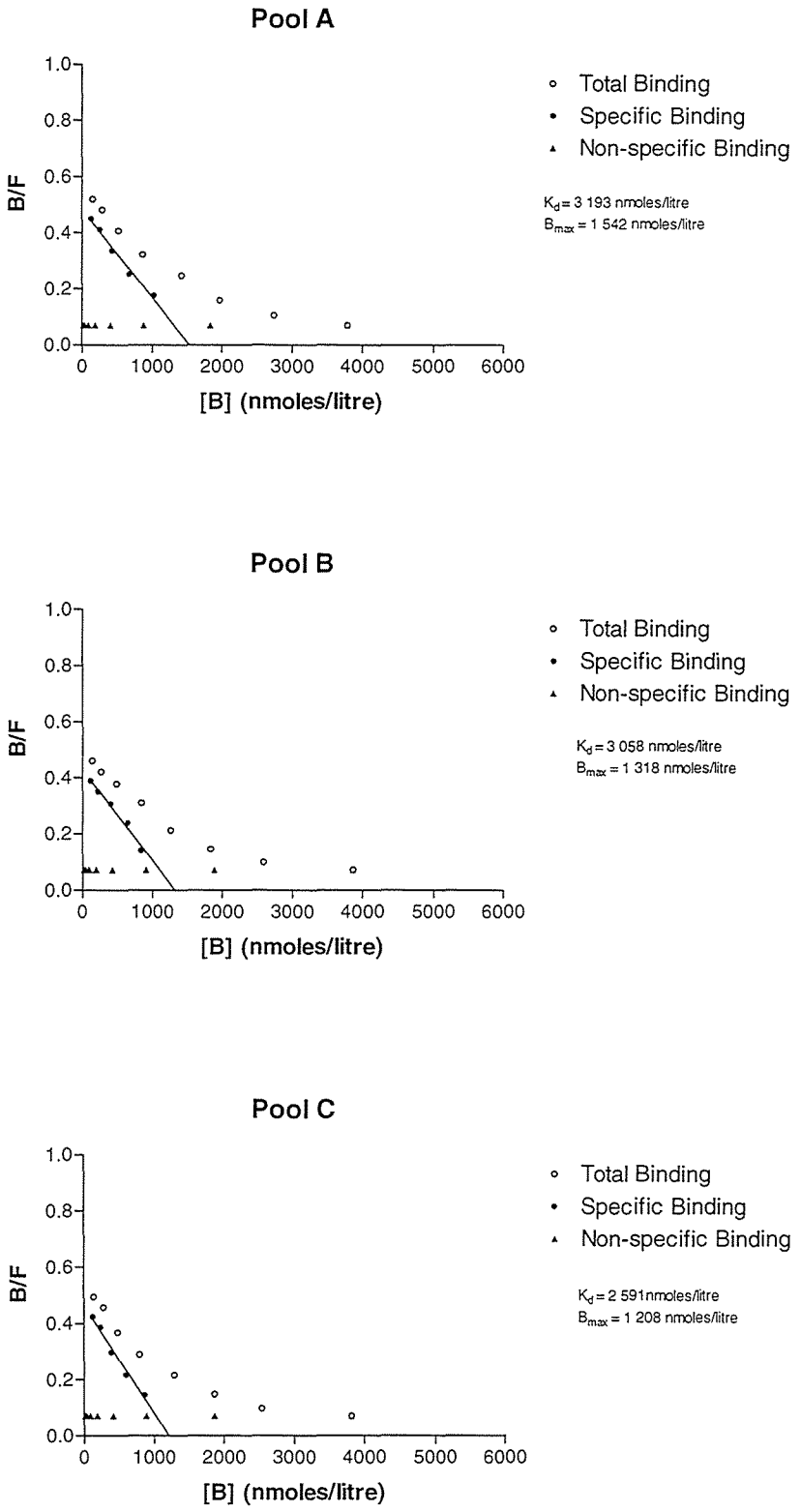


Figure 4.4.15 Scatchard plots for the binding of corticosterone to CBG for three pools of plasma collected from nocturnal house kiwi. K_d and B_{max} for each experiment are indicated in the legends.

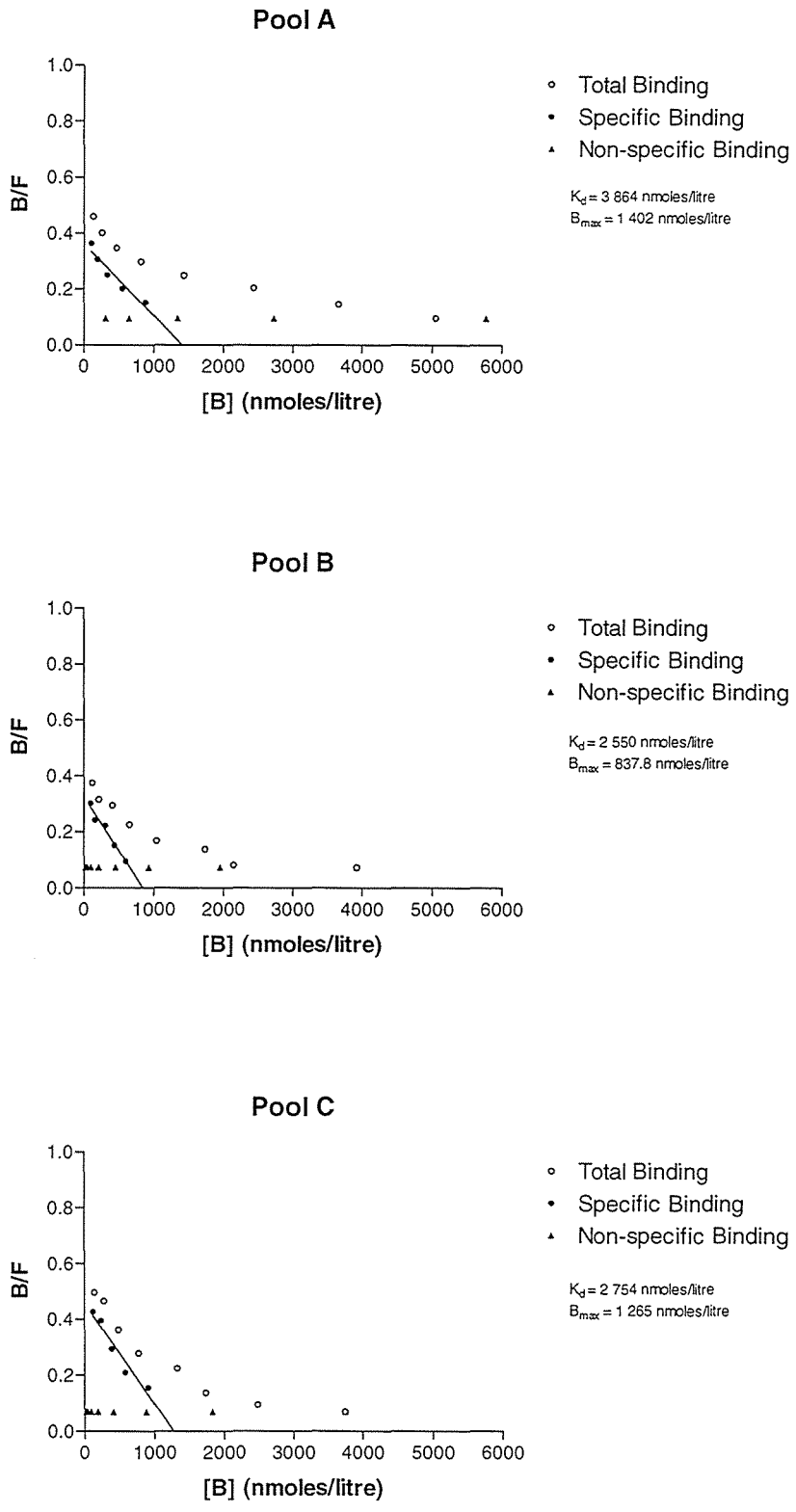


Figure 4.4.16 Scatchard plots for the binding of corticosterone to CBG for three pools of plasma collected from outdoor penned kiwi. K_d and B_{max} for each experiment are indicated in the legends.

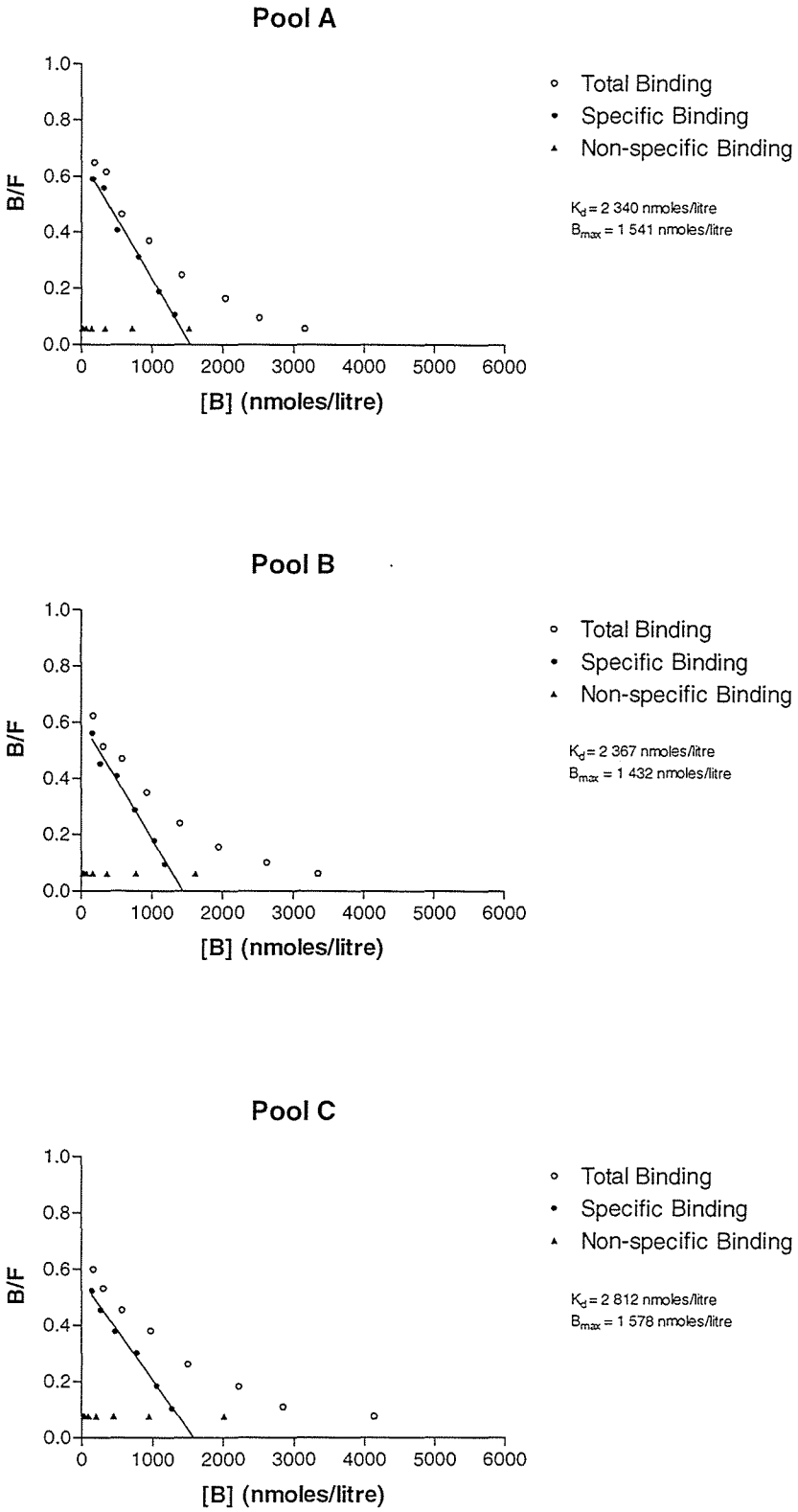


Figure 4.4.17 Scatchard plots for the binding of corticosterone to CBG for three pools of plasma collected from wild kiwi. K_d and B_{max} for each experiment are indicated in the legends

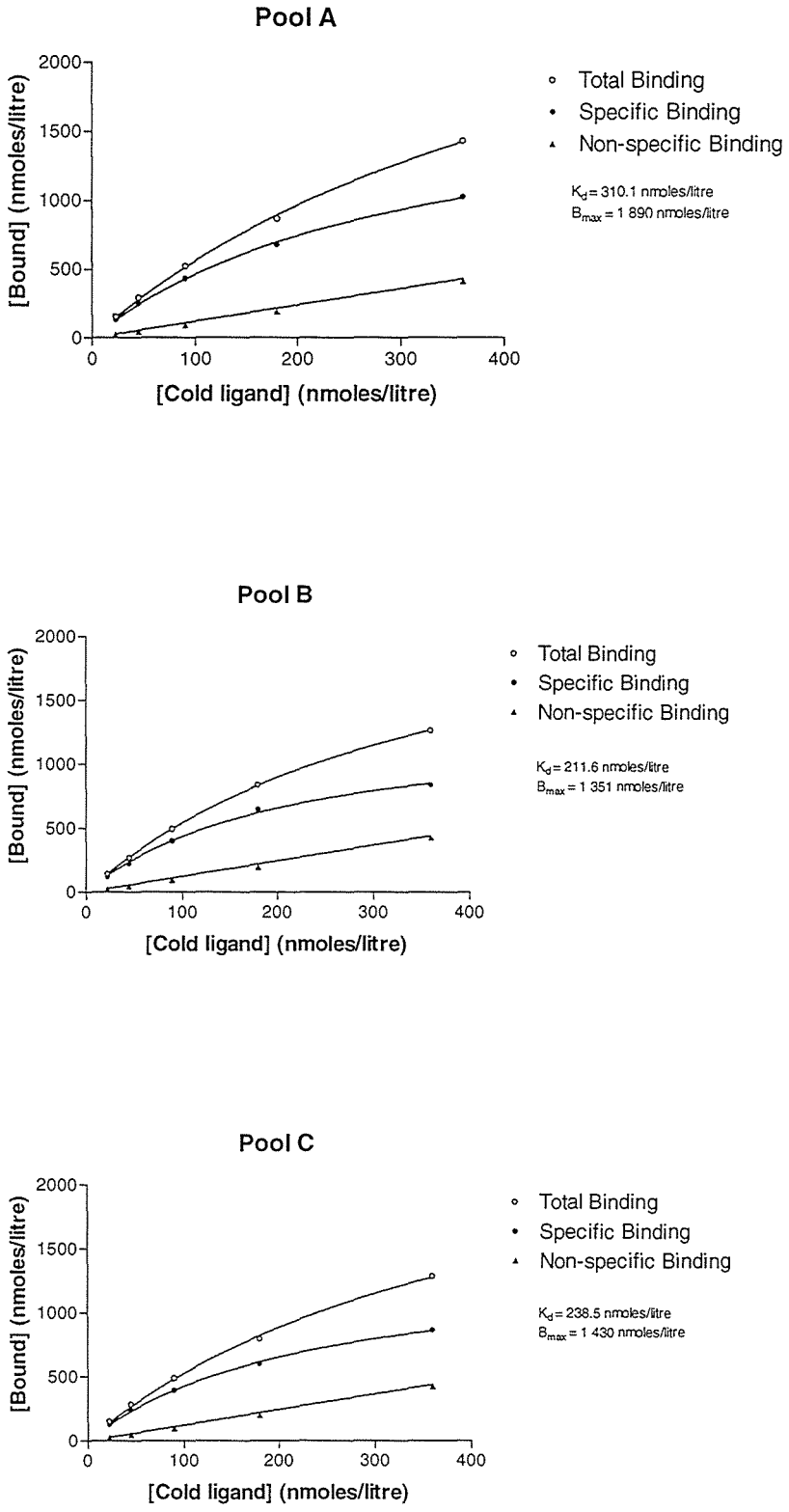


Figure 4.4.18 Non-linear regression plots for the binding of corticosterone to CBG for three pools of plasma collected from nocturnal house kiwi. K_d and B_{max} for each experiment are indicated in the legends.

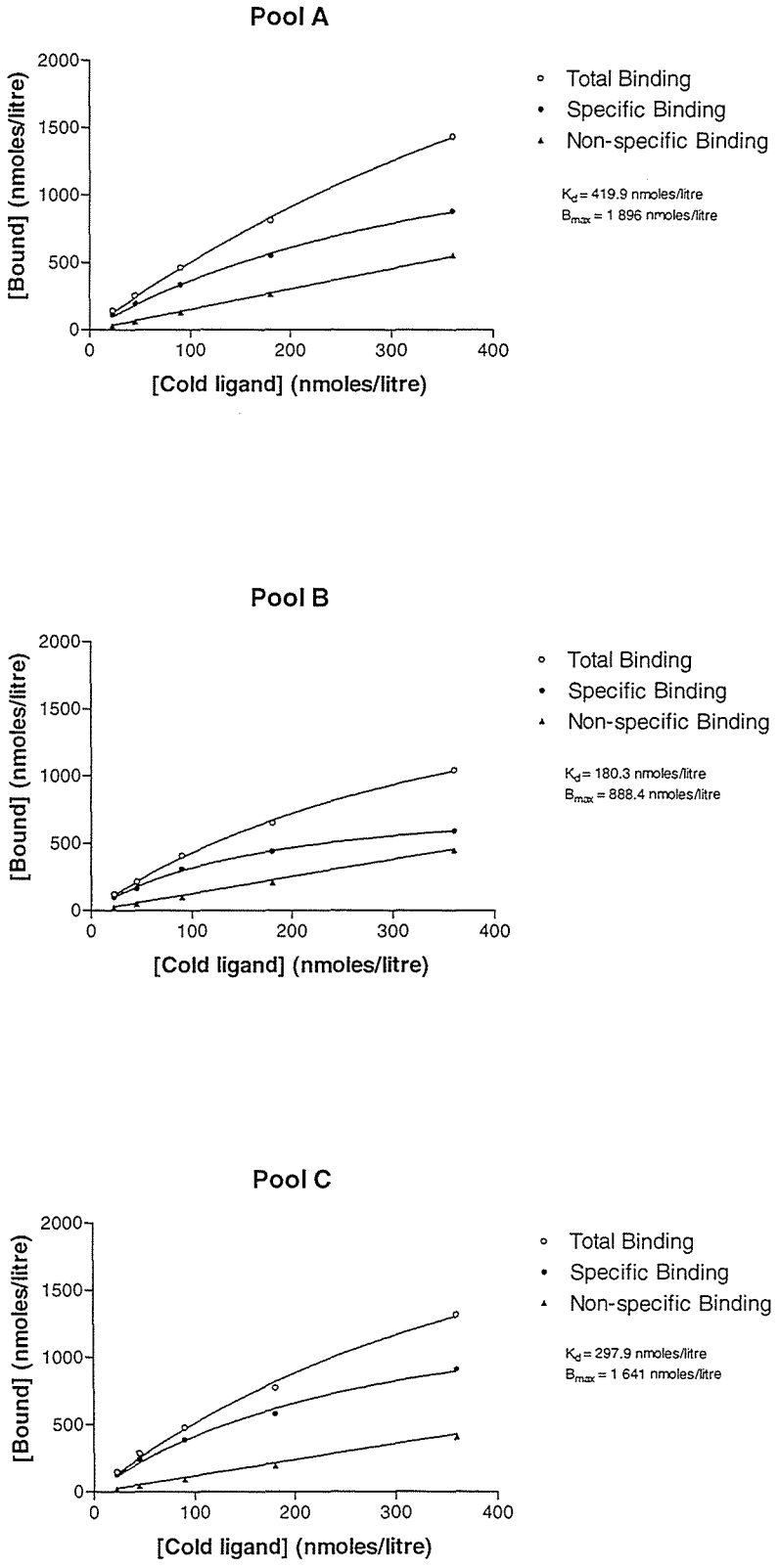


Figure 4.4.19 Non-linear regression plots for the binding of corticosterone to CBG for three pools of plasma collected from outdoor penned kiwi. K_d and B_{max} for each experiment are indicated in the legends.

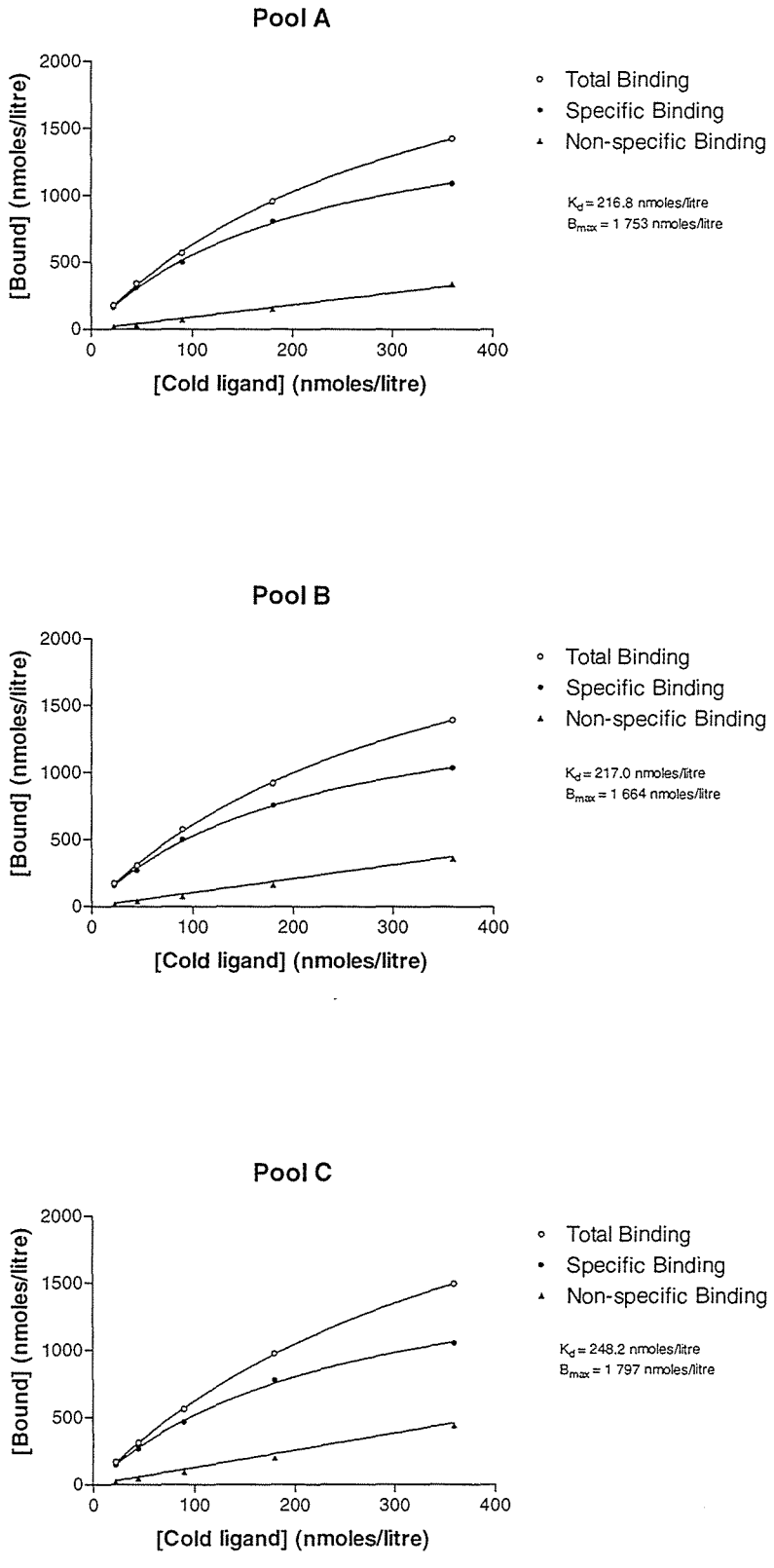


Figure 4.4.20 Non-linear regression plots for the binding of corticosterone to CBG for three pools of plasma collected from wild kiwi. K_d and B_{max} for each experiment are indicated in the legends.

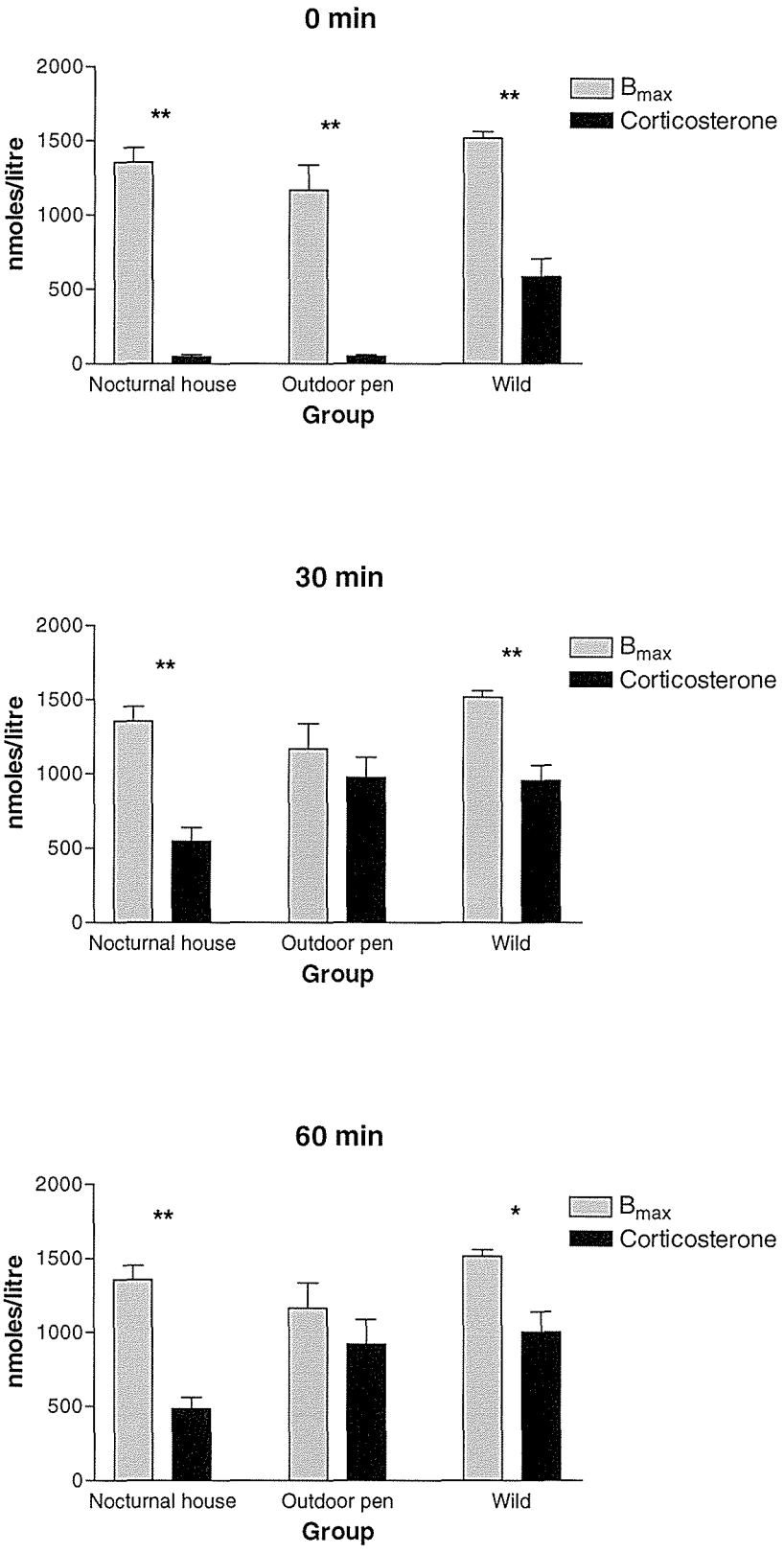


Figure 4.4.21 Comparison of corticosterone titres and maximum binding capacity of CBG in kiwi. **Significant difference ($P<0.001$) between B_{max} and corticosterone, *Significant difference ($P<0.05$) between B_{max} and corticosterone. All values are means ± S.E.M.

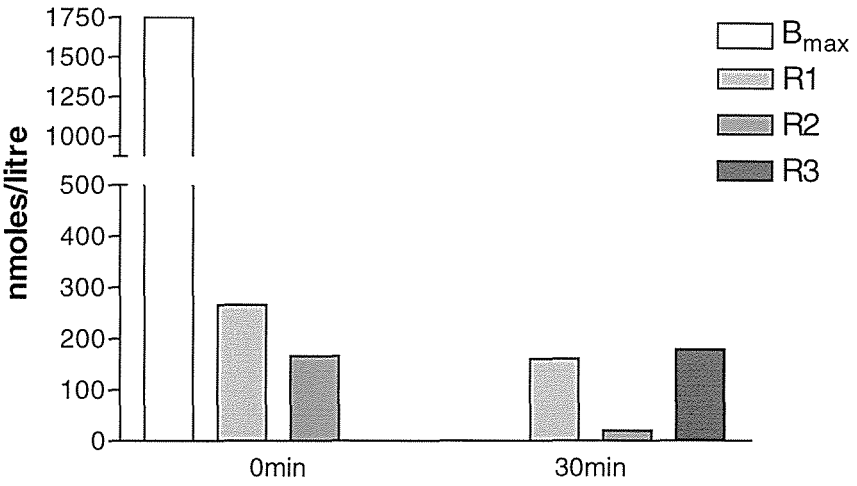


Figure 4.4.22 Comparison of individual corticosterone values with B_{max} for regularly handled kiwi before (0 min) and after (30 min) a period of public display.

Table 4.4.3 Comparison of Scatchard and non-linear regression analysis in determining K_d and B_{max} for each group of kiwi and quail. Coefficients of variation (CV) are also indicated in the table for each group.

	Scatchard plot analysis				Non-linear regression analysis			
Group	**K_d (nM)**	**% CV**	**B_{max} (nM)**	**% CV**	**K_d (nM)**	**% CV**	**B_{max} (nM)**	**% CV**
Nocturnal house kiwi (n=3)	2947 ± 182	6.19	1356 ± 98	7.25	253 ± 29	11.60	1557 ± 168	10.79
Outdoor pen kiwi (n=3)	3056 ± 408	13.36	1168 ± 170	14.54	299 ± 69	23.11	1475 ± 302	20.50
Wild kiwi (n=3)	2506 ± 153	6.11	1517 ± 44	2.89	227 ± 10	4.59	1738 ± 39	2.25
Regularly handled kiwi (n=1)	2158	—	1749	—	227	—	2060	—
Short day male quail (n=3)	401 ± 16	4.02	108 ± 10	8.82	24 ± 1	4.26	106 ± 9	8.75

Note: K_d and B_{max} values are mean ± S.E.M.

4.5 Discussion

4.5.1 Experiment 1. Verifying methodology using pooled quail plasma

This experiment confirms the presence of a high affinity (K_d ~10^{-8} M), low capacity (B_{max} ~10^{-8} M) binding protein for corticosterone in the plasma of quail (corticosterone binding globulin, CBG). The values measured here are similar to those determined previously for Japanese quail and for many other species of bird (refer to Appendix 1). Clear regions of specific (S) and non-specific (NS) binding portions of the total binding curve (Scatchard analysis) confirm that the CBG molecule measured here consists of a one site binding system. Furthermore, the presence of a straight line for NS binding validates the presence of a low affinity and high capacity NS binding system, most likely due to plasma albumins (Siiteri et al., 1982; Wingfield et al., 1984). Non-linear regression analysis of the same data also demonstrated clear regions of S and NS binding.

Typically, competitive binding data in avian species have been analysed using Scatchard analysis (refer to Appendix 1). However, this method is inaccurate as it violates some of the underlying assumptions of linear regression. Therefore, non-linear regression analysis should be used with this type of data. To compare these results with other studies however, it is necessary to use both analyses. Analysing the data from this experiment with Scatchard analysis and non-linear regression analysis resulted in similar determinations of B_{max}. However, these two analyses differed in their determination of K_d, with non-linear regression giving an approximately 20-fold increase in binding affinity. The cause of this difference is not known.

In this experiment, the K_d and B_{max} was determined for the same plasma pool three times. The coefficient of variation for this experiment was less than 10%, suggesting that this procedure could be repeated accurately.

4.5.2 Experiment 2. Testing of methodology using pools of kiwi plasma

Scatchard analysis of these data showed that there were no clear regions of S and NS binding in kiwi plasma. The cause of this apparent lack of S binding is unclear without non-linear regression analysis. Cold and hot ligand do not compete for NS binding sites due to their very high capacity, therefore binding with equal rates (Clark and Peck Jr, 1977). Non-linear regression analysis showed that both S and NS binding sites were in fact binding hot and cold ligands at equal rates. This suggests that hot and cold ligands were not in solution at high enough concentrations to compete for the high affinity and low capacity S binding sites. Therefore, for all further experiments to determine K_d and B_{max} for kiwi, an increase in the concentration of cold ligand was required to compete with hot ligand for the high affinity S binding sites.

4.5.3 Experiment 3. The effect of varying concentrations of hot and cold competing ligand

Increasing the concentration of competing cold ligand resulted in discernible S and NS binding regions of the total binding curve for kiwi plasma (Scatchard analysis). This confirmed the presence of a high affinity and low capacity (with respect to the NS binding sites) one site binding system (CBG). However, the binding affinity of kiwi CBG (K_d ~10^{-6} M) for corticosterone was a lot lower than has been reported for other species (refer to Appendix 1). Furthermore, the capacity of kiwi CBG (B_{max} ~10^{-6} M) was also much larger than reported for any other species (refer to Appendix 1). The importance of these features of kiwi CBG will be discussed further in section 4.5.5.

Decreasing the amount of hot ligand had little influence on K_d or B_{max} for kiwi CBG. However, the specific binding sites of kiwi CBG were not saturated when the concentrations of both hot and cold ligand were reduced further. This was confirmed with non-linear regression analysis, where S and NS curves increased at similar rates. Therefore, the ideal amounts of hot and cold competitor to measure K_d and B_{max} of kiwi CBG are 10 000 cpm and 1 000 ng/ml respectively.

Large concentrations of cold competitor (with respect to the B_{max} of the specific receptor in question) can result in the alteration of S and NS binding (Clark and Peck Jr, 1977). An excess of cold competitor can out-compete the hot ligand for S binding sites. This can result in the overestimation of NS binding and the underestimation of S binding (Westphal, 1971; Clark and Peck Jr, 1977). In this experiment, various quantities of competing hot and cold ligand were used to measure quail CBG. Non-linear regression analysis revealed that when excess cold ligand was present in solution, S binding began to drop as the cold ligand began to displace all hot ligand from S binding sites. This would therefore overestimate NS binding, seen in non-linear regression analysis as the NS binding curve increases at a greater rate than the S binding curve. Reducing the amount of hot and concentration of cold competitor resulted in distinct S and NS binding regions of the total binding curve. Therefore, the effect of excess cold ligand seen here was not just an artefact of the pool of plasma used in this experiment.

4.5.4 Experiment 4. The effect of plasma stripping method on corticosterone concentration, K_d and B_{max} of kiwi and quail plasma

4.5.4.1 Effect of temperature

The stripping of endogenous steroid from plasma with DCC was conducted in this study initially at 4°C overnight. However, this method was found to only strip 60% of endogenous corticosterone from kiwi plasma. In all other studies of avian, mammalian and amphibian species, stripping of endogenous steroid with DCC is performed at approximately 20-45°C for periods of up to 2 h (Gould and Siegel, 1978a; Wingfield et al., 1984; Wingfield et al., 1992; Klukowski et al., 1997; Taymans et al., 1997; Boonstra and Tinnikov, 1998; Romero et al., 1998; Orchinik et al., 2000). The binding affinity of a receptor for its ligand (e.g. CBG for corticosterone) is known to be temperature dependent, with the binding affinity decreasing as the temperature increases (Siiteri et al., 1982; Hammond, 1995). Therefore, by increasing the temperature at which the plasma is stripped with DCC should result in more endogenous steroid being removed. Increasing the temperature during stripping of kiwi plasma did result in more endogenous steroid being removed (75% at 20°C and 93% at 36°C). The removal of

approximately 93% of endogenous corticosterone in kiwi plasma is similar to that found in chickens (~ 97%, Gould and Siegel, 1978). It appears though, that measuring the steroid content of stripped plasma is an uncommon practice by researchers in this field. In addition, increasing the amount of steroid removed from solution had no effect on the K_d and B_{max} of kiwi CBG.

In quail plasma, increasing the temperature during stripping had no effect on the amount of corticosterone removed from the plasma. The K_d and B_{max} of quail CBG were also unaffected by an increase in temperature to 20°C during stripping. However, increasing the temperature to 36°C resulted in a significant drop in S binding in quail plasma, which was not demonstrated in kiwi plasma. The cause of this drop in S binding using Scatchard analysis was unclear. However, non-linear regression analysis reveals that this was probably caused by non-competitive inhibition. This was demonstrated in graphical form by straight parallel lines for both total binding and NS binding, and the almost complete lack of S binding. Non-competitive inhibition can be due to an inhibitor causing the partial precipitation of the receptor, or an inhibitor binding to a second site on the CBG molecule and in doing so altering the receptor binding site (Clark and Peck Jr, 1977). However, both of these explanations are unlikely to be caused by an increase in temperature during stripping. It also seems unlikely that the increase in temperature to 36°C denatured the CBG molecule (which is a protein), as the normal body temperature of kiwi is ~37°C and other birds are between 38°C and 40°C. The function of dextran in DCC is to bind to the larger binding sites on the charcoal (Herbert et al., 1965). The dextran used in this experiment has a molecular weight of ~70 kDa, and the molecular weight of avian CBG was determined by Gould and Siegel (1978) as ~55 – 63 kDa. Therefore, exposure of quail CBG to DCC at the higher temperature for two hours may explain the decrease in S binding in quail plasma, due to absorption of CBG by DCC. A similar interpretation may also explain the reduction in B_{max} of chicken plasma after stripping (Gould and Siegel, 1978a). Kiwi plasma also has a considerably higher concentration of CBG than quail plasma, therefore exposure to DCC for this length of time probably did not remove a significant proportion of CBG in kiwi plasma. This may explain the presence of S binding in kiwi plasma after stripping for 2 h at 36°C. Therefore, all further stripping of kiwi plasma was conducted at 36°C,

for 1 h. This method will increase the amount of endogenous steroid absorbed, without absorbing a significant proportion of CBG molecules.

4.5.4.2 Effect of DCC addition method

There was little difference in K_d and B_{max} between the two methods used to strip plasma (adding DCC directly to plasma and adding a DCC-buffer mix). However, corticosterone levels in plasma stripped by direct DCC addition to plasma were slightly higher than those stripped by the DCC-buffer solution.

4.5.5 Experiment 5. Measurement of K_d and B_{max} in nocturnal house, outdoor pen, wild and regularly handled kiwi

In several avian species, plasma concentrations of CBG have been shown to vary between seasons. For example, B_{max} was significantly reduced during the nestling period in pied flycatchers (*Ficedula hypoleuca*) (Silverin, 1986), and during moult in Lapland longspurs (*Calcarius lapponicus*) (Romero et al., 1998) and white crowned sparrows (*Zonotrichia leucophrys gambelli*) (Romero and Wingfield, 1998). Circulating concentrations of CBG may also exhibit a circadian pattern (Siegel et al., 1976; Kovacs and Peczely, 1983). Furthermore, these seasonal and diurnal changes in CBG are often correlated closely with changes in basal and stress induced plasma corticosterone levels. For example, the increase in plasma levels of corticosterone in white crowned sparrows and Lapland longspurs during the breeding season (compared with moulting birds) are associated with concurrent rises in CBG concentration (Romero et al., 1998; Romero and Wingfield, 1998). In the Japanese quail, the circadian rhythm in plasma corticosterone concentration is closely mirrored by circadian changes in CBG concentration (Kovacs and Peczely, 1983). However, seasonal variations in basal and stress induced plasma corticosterone levels may not necessarily be exhibited by their captive counterparts (e.g. Romero et al., 1998; Breuner et al., 1999; Romero and Wingfield, 1999). Moreover, it has yet to be investigated whether CBG titres may also vary between captive and wild conspecifics.

Wild kiwi had higher plasma corticosterone levels after exposure to a stressor than nocturnal house kiwi (refer to chapter 3). However, this difference may not be biologically meaningful if the increase in corticosterone release was associated with a concurrent rise in B_{max}, thereby making the amount of free corticosterone equivalent between the two groups. However, B_{max} and K_d did not differ between captive (nocturnal house, outdoor pen) and wild kiwi. Furthermore, at no time did corticosterone titres exceed the B_{max} of kiwi plasma. Only in outdoor penned kiwi 30 min and 60 min after capture was B_{max} saturated by plasma levels of corticosterone. However, this result is greatly influenced by small sample size (n=3) and a single very low value for B_{max}. The single determination of K_d and B_{max} of CBG in regularly handled kiwi was within the range of values of the other three groups of kiwi. Furthermore, plasma levels of corticosterone before and after a period of public display were significantly less than the maximum binding capacity of CBG.

Initially these results seem similar to those reported by Wingfield et al. (1992) for four species of bird found on the Sonoran desert. Wingfield et al. (1992) reported that at no time did maximum corticosterone titres exceed the B_{max} for any of the species studied. They concluded, (that) "...binding capacity for corticosterone was not a major factor when interpreting the responses of circulating corticosterone to capture stress". However, closer examination of their results indicates that maximum corticosterone titres generated during capture stress did in fact greatly exceed the B_{max}. In contrast to Wingfield et al.s' (1992) study, it can be concluded that the binding capacity of CBG in kiwi plasma was not a major factor when interpreting corticosterone responses of kiwi in the present study.

The biochemistry and endocrinology of CBG and corticosterone in kiwi are very unusual for an avian species. Firstly, the binding affinity of kiwi CBG is considerably weaker than in other avian species (refer to Appendix 1). Secondly, the binding capacity is significantly larger than has been previously recorded for any avian species (refer Appendix 1). Furthermore, the highest recorded corticosterone titres induced during capture and handling in other species of bird range from 100 – 150 ng/ml (refer to chapter 3, and references cited therein). However, in the kiwi, peak corticosterone levels induced during capture and handling ranged from 150 – 660 ng/ml, while basal levels were similar to other avian species. Therefore, in avian species there appears to

be two types of corticosteroid binding systems. All other avian species studied thus far have low concentrations of corticosterone in response to a stressor and high affinity, low capacity CBG. Another system, seen here with kiwi, has high concentrations of corticosterone in response to a stressor and low affinity, high capacity CBG.

The novel situation of unusually high levels of corticosteroids (both basal and stress induced) associated with low binding affinities of CBG has been demonstrated in prairie voles (*Microtus ochrogaster*), and is characteristic of 'glucocorticoid resistance' (Taymans et al., 1997). Glucocorticoid resistance is further characterised by decreased target tissue sensitivity, due to a decrease in tissue glucocorticoid receptor (GR) density and affinity (Chrousos et al., 1993). This is associated with a compensatory hypersecretion of glucocorticoids, and high concentrations of low affinity binding proteins, without the normally associated detrimental effects of chronic elevations of glucocorticoids (Chrousos et al., 1993). In prairie voles, both basal and stress induced titres of corticosterone are approximately four fold higher than in the closely related montane vole (*Microtus montanus*) and Sprague-Dawley rat (Taymans et al., 1997). However, there were no associated detrimental effects of elevated corticosterone. Further investigation revealed that there was a decrease in abundance of GR in prairie vole liver cytosol, and a decreased affinity of corticosteroid receptors in the liver and whole brain cytosol. Taymans et al. (1997) conclude that this might contribute to glucocorticoid resistance.

These characteristics of high corticosterone levels in response to a stressor and low affinity plasma binding proteins (CBG) are also seen in kiwi. Furthermore, kiwi appear to function perfectly well in captivity and the wild with transient high levels of corticosterone. However, kiwi have very low basal levels of corticosterone, which is not characteristic of glucocorticoid resistant mammals. Although these results cannot conclude glucocorticoid resistance in kiwi, they do suggest that kiwi are the first avian species to be found with biochemical and endocrine characteristics similar to those of glucocorticoid resistant mammals. To further explain the biological significance of very high plasma corticosterone levels in response to a stressor, and low affinity binding proteins in kiwi, GR density, affinity and distribution in various target tissues should be investigated.

4.5.6 Conclusions

The protocol for measuring CBG was validated using quail plasma by confirming the presence of a high affinity, low capacity, one site plasma protein binding system for corticosterone (CBG). The optimal conditions for the measurement of quail CBG K_d and B_{max} were to strip the plasma at 20°C for 2 h, and then compete 100 ng/ml cold and 10 000 cpm hot ligand together in solution. This method was found to be generally repeatable. Scatchard analysis and non-linear regression analysis did not differ in their determinations of B_{max}. However, there was a 20 fold increase in binding affinity of quail CBG when analysed with non-linear regression, compared with Scatchard analysis.

Increasing the temperature during stripping of endogenous steroid from the plasma resulted in an increase in the amount of corticosterone removed from kiwi plasma. Conversely, increasing the temperature for quail plasma resulted in a significant reduction in specific binding, and no change in the amount of corticosterone removed.

The optimal conditions for the measurement of kiwi CBG K_d and B_{max} were to strip the plasma at 36°C for 1 h, and then compete 1 000 ng/ml cold and 10 000 cpm hot ligand together in solution. This methodology was found to be generally repeatable. Furthermore, the binding characteristics were considerably different to all other avian species studied to date.

At no stage did plasma corticosterone concentrations exceed the maximum binding capacity of kiwi CBG. Therefore, the binding capacity of kiwi CBG is not a major factor when interpreting corticosterone responses of kiwi.

5 General discussion

The capture stress protocol (CSP) was an effective means of comparing the sensitivity of the hypothalamic pituitary adrenal (HPA) axis of kiwi in different management systems. All kiwi responded to capture and handling with an increase in corticosterone, however there was considerable variation between individuals in the magnitude and duration of the response, similar to that found in other species. Furthermore, there seemed to be no effect of gender on the corticosterone response.

An inherent problem with this study was sample size, with some samples consisting of just three individuals. Although statistical analyses were performed using methods that account for sample size (e.g. Bonferroni post hoc comparisons), these results were interpreted with caution. This often made it difficult to make definitive conclusions.

5.1 The corticosterone responses of kiwi in different management systems

In captivity, it was possible to quickly catch the kiwi and remove a blood sample. This allowed an accurate determination of basal levels of corticosterone. Conversely, capture of wild kiwi using radio-telemetry equipment is difficult. Immediately after capture, plasma corticosterone levels of wild kiwi were considerably higher than captive kiwi basal levels, suggesting that wild kiwi were aware of our presence 5 to 18 min prior to capture. This has important implications for field management of kiwi, and for any other species. It would seem that trying to locate the position of kiwi in the wild is sufficient to induce a stress response. Therefore, determining the precise location of wild kiwi should be kept to a minimum so as to avoid the possible deleterious effects of frequent and chronic elevations of corticosterone. This finding also highlights the importance of not underestimating the effect of observers on the behaviour and physiology of study species (this is discussed in depth by Hofer and East, 1998).

Nocturnal house kiwi responded to capture and handling with a significantly lower corticosterone response than wild kiwi. The cause of this difference is unclear. It is possible that nocturnal house kiwi have become habituated to the presence of humans, and thus responded to capture and handling with a reduced adrenocortical response. Alternatively, a possible seasonal variation in the sensitivity of the HPA axis may explain the difference. Furthermore, it is conceivable that both possibilities were acting in concert to produce the reduced adrenocortical response to capture and handling by nocturnal house kiwi. Importantly, neither nocturnal house nor outdoor pen kiwi displayed an increase in the sensitivity of the HPA axis with respect to wild kiwi. Although this does not prove the absence of stressful captive management systems, it does provide evidence that captive kiwi are not subjected to the deleterious effects of chronic elevations in corticosterone.

5.2 The effect of regularly handling kiwi for public display

Recently there has been considerable debate as to whether kiwi should be regularly handled for display to the public. This study found that regularly handled kiwi do not respond to a period of public display with the classic non-specific response to stress, an increase in plasma levels of corticosterone. However, regularly handled kiwi did have higher basal levels of corticosterone than non-regularly handled kiwi. This should be interpreted with caution due to the very small sample size (n=2). Although this study provides convincing evidence that kiwi, as with all other species, can become habituated to handling, it does not answer the question of whether kiwi should be handled for public display. The ethical considerations of using kiwi for public display have yet to be addressed. This study does, however, provide objective scientific data for this discussion.

5.3 Corticosteroid binding globulin and corticosterone in kiwi

A method for measuring corticosteroid binding globulin (CBG) was validated using plasma from Japanese quail. These results were similar to those found previously for Japanese quail (Wingfield et al., 1984), and proved to be generally repeatable. A series

of experiments determined the optimal conditions for measuring K_d and B_{max} for kiwi CBG. Determinations of K_d and B_{max} for kiwi CBG are important, as CBG can significantly affect the circulating concentration of corticosterone, and its bio-availability. This study found no difference between nocturnal house, outdoor pen and wild kiwi in the binding affinity and capacity of CBG. Furthermore, at no stage did circulating corticosterone titres exceed the maximum binding capacity of kiwi CBG. Therefore, CBG was not a major factor when interpreting corticosterone responses of kiwi.

The biochemistry of CBG and endocrinology of corticosterone in kiwi are unique for an avian species. Kiwi have considerably higher circulating levels of corticosterone than has been reported for any other avian species. Furthermore, the binding capacity of CBG is considerably higher, while the binding affinity is considerably lower than any other avian species. Therefore, there appears to be at least two mechanisms that underlie the bio-availability of corticosterone in birds. Kiwi are the first avian species described with biochemical and endocrine characteristics similar to those of glucocorticoid resistant mammals (Chrousos et al., 1993; Taymans et al., 1997).

5.4 Directions for future research

This study found no effect of gender on the corticosterone response. However, kiwi were sampled during the non-breeding season. It would be interesting to sample kiwi during the breeding season, as this is the time when a sexual dimorphism in the corticosterone response, if it exists at all, is most often revealed in other species (Wingfield et al., 1998). Furthermore, sampling of wild kiwi at different stages during the breeding season would shed light on the behavioural observations that kiwi have an increased level of tolerance to disturbance during the latter part of incubation.

These results also hint at a possible seasonal effect on the corticosterone response of nocturnal house kiwi. If this proves to be so, then it is likely that other endogenous rhythms such as the reproductive cycle will be likewise affected. This should be investigated further by comparing the annual cycle of gonadal steroids (perhaps via a non-invasive faecal steroid analysis) in captive kiwi with those already established for

wild kiwi. Such an investigation may help explain the lower breeding success of nocturnal house kiwi compared to outdoor pen and wild kiwi.

Captive kiwi weigh significantly less than wild kiwi. This difference in body weight appears to be generally unrelated to corticosterone levels. An investigation into the nutritional value of the diet fed to captive kiwi would be necessary to explain the difference in body weights.

During the course of this study, it was often difficult to make definitive conclusions due to small sample sizes. Therefore, increased sample size would be necessary to further investigate what effect, it any, the Willowbank and outdoor pen with public access management systems have on the corticosterone responses of kiwi. Likewise, a larger sample size would be necessary to clarify the high basal levels of corticosterone in regularly handled kiwi. Alternatively, this could also be achieved by repeated sampling of the same individuals over a longer period using faecal steroid analysis.

In comparison to other birds, kiwi have very high plasma levels of corticosterone. Furthermore, kiwi CBG is of low affinity and very high capacity. To further clarify the biological significance of these characteristics, it would be necessary to describe the density, affinity and distribution of glucocorticoid receptors in various target tissues of kiwi.

In summary, the CSP was an effective means of comparing the sensitivity of the HPA axis of kiwi in different management systems. There was no difference in the K_d and B_{max} of CBG between nocturnal house, outdoor pen and wild kiwi. Therefore, CBG was not a major factor when interpreting the corticosterone responses of kiwi. This study also demonstrated that trying to determine the precise location of radio-tagged kiwi in the wild can induce a stress response. Furthermore, captive kiwi appear not be subjected to chronic elevations in corticosterone. This study also found that regularly handled kiwi do not respond to handling for public display with a rise in plasma levels of corticosterone. Therefore, this study provides important information for the further development and refinement of management protocols for captive and wild northern brown kiwi.

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Appendix 1

Appendix 1 Summary of previously recorded values for K_d and B_{max}. All analyses were conducted using the Scatchard method.

Species	K_d (nM)	B_{max}(nM)
Results from this study:		
Nocturnal house kiwi	2947 ± 182	1356 ± 98
Outdoor pen kiwi	3056 ± 408	1168 ± 170
Wild kiwi	2506 ± 153	1517 ± 44
Regularly handled kiwi	2158	1749
Quail (short day male)	400 ± 16	108 ± 10
From Wingfield et al. (1984)		
Quail (male)	84.4	73.5
Quail (female)	40.2	6.46
From Wingfield et al. (1992)		
Inca dove (male)	0.0054	32.21
Inca dove (female)	0.0056	18.79
Abert's towhee (male)	0.0079	9.44
Abert's towhee (female)	0.077	7.45
Black-throated sparrow (male)	0.0042	4.43
Curve-billed thrasher (male)	0.0057	4.61
From Romero et al. (1998a)		
Snow bunting (breeding)	639.0	
Snow bunting (moult)	643.0	
From Romero and Wingfield (1998)		
Gambel's White –Crowned Sparrow (breeding)	525.0	
(winter)	482.0	
(migration)	460.0	
From Romero et al. (1998c)		
Redpolls (at Toolik lake)	456.0	
(at Barrow)	794.0	
(during moult)	676.0	
From Romero et al. (1998b)		
Lapland Longspur (Breeding)	490.0	
(Moult)	500.0	

Appendix 1 continued:

Species	K_d (nM)	B_{max}(nM)
From Silverin (1986)		
Pied Flycatcher (pre-nesting male)	25.4	117.0
(pre-nesting female)	7.8	43.0
(nesting male)	15.2	8.0
(nesting female)	10.1	17.0
From Kovacs and Peczely (1983)		
Japanese Quail		0.94
From Klukowski et al. (1997)		
Dark-eyed Juncos		76.6
From Wingfield et al. (1984)		
White-crowned sparrow	2.43	75.8
Larus occidentalis	44.7	35.5
Columbia livia	478.0	77.1
Gallus domesticus	55.9	14.4
Anas platyrhynchos	7.52	2.91