CHAPTER 2

GENERAL METHODS

2.1 Lion-tailed macaque subjects and housing

Subjects were 38 captive lion-tailed macaques (*Macaca silenus*), housed in four groups, one each at The North of England Zoological Society (referred to from this point on as Chester Zoo), Bristol Zoological Gardens (referred to from this point on as Bristol Zoo), Assiniboine Park Zoo and San Diego Wild Animal Park (see appendix 1 for institution addresses). The subjects were divided into age groups (table 2.1) for analysis based on previous research (Green & Minkowski, 1977; Lindburg, 2001; Rowe, 1996). Details on subjects at Chester Zoo are shown in table 2.2, Bristol Zoo in table 2.3, Assiniboine Park Zoo in table 2.4 and San Diego Wild Animal Park in table 2.5. One individual at the San Diego Wild Animal Park was vasectomised and housed alone. This individual was only included in one part of the current research (see chapter 3 section 3.2.5 for details).

Table 2.1 – *Age groups for male and female lion-tailed macaque subjects.*

Age group	Age (years)
Infant	0-1.5
Juvenile Female	1.5-5
Juvenile Male	1.5-8
Adult Female	5+
Adult Male	8+

Table 2.2 – *Details of lion-tailed macaque subjects at Chester Zoo. An infant was born during the study period increasing the group size to eight. However data was not collected on this individual so it is not included in the table.*

Subjects	Sex	Date of birth	Age at start of observation (years/months)	Age class	Place of birth	Sire / Dam	Rearing	Samples collected
Jam	Male	23/03/95	8y 3m	Adult	Belfast	1429/1785	Mother	Urine/Faeces
Rem	Female	13/12/92	10y 6m	Adult	Edinburgh	1703/1678	Mother	Urine/Faeces
Tin	Female	29/01/95	8y 5m	Adult	Colchester	1190/1623	Mother	Urine/Faeces
Hub	Female	01/09/99	3y 9m	Juvenile	Chester	Jam/Rem	Mother	Urine/Faeces
Tia	Female	03/03/01	2y 3m	Juvenile	Chester	Jam/Rem	Mother	Urine/Faeces
Spo	Male	07/03/01	2y 3m	Juvenile	Chester	Jam/Tin	Mother	Urine/Faeces
Mug	Female	06/09/02	0y 9m	Infant	Chester	Jam/Tin	Mother	Urine/Faeces

Table 2.3 – *Details of lion-tailed macaque subjects at Bristol Zoo*

Subjects	Sex	Date of birth	Age at start of observation (years/months)	Age class	Place of birth	Sire / Dam	Rearing	Samples collected
Dam	Male	13/02/91	13y 11m	Adult	St Catherine's Island	730/1639	Mother	Faecal
Kil	Female	08/12/89	15y 1m	Adult	Bristol	1782/1500	Mother	Faecal
Cas	Female	27/01/92	13y 0m	Adult	Bristol	1536/1500	Mother	Faecal
Her	Male	05/12/01	3y 11m	Juvenile	Bristol	Dam/Cas	Mother	Faecal
Holl	Female	13/01/02	3y 0m	Juvenile	Bristol	Dam/Kil	Mother	Faecal
Jame	Female	17/01/04	1y 0m	Infant	Bristol	Dam/Cas	Mother	Faecal

Table 2.4 – *Details of lion-tailed macaque subjects at Assiniboine Park Zoo. *Faecal samples were not collected from individuals Nag, Jam and Jag because they were too young to individually hand-feed (see section 3.3).*

Subjects	Sex	Date of birth	Age at start of observation (years/months)	Age class	Place of birth	Sire/Dam	Rearing	Samples collected
Jal	Female	21/06/85	19y 11m	Adult	Assiniboine	910076/970140	Mother	Faecal
Mar	Male	04/06/95	9y 11m	Adult	Toronto	20487/25482	Hand	Faecal
JoJ	Female	15/02/97	8y 3m	Adult	Assiniboine	910088/Jalna	Mother	Faecal
MoJ	Female	23/04/97	8y 1m	Adult	Assiniboine	910088/910079	Mother	Faecal
Jet	Female	03/08/98	6y 9m	Adult	Assiniboine	910088/Jalna	Mother	Faecal
Mon	Male	03/12/00	4y 5m	Juvenile	Assiniboine	910088/910079	Mother	Faecal
Jas	Male	04/03/02	3y 2m	Juvenile	Assiniboine	Mar/Jal	Mother	Faecal
Kiz	Female	17/03/02	3y 2m	Juvenile	Assiniboine	Mar/JoJ	Mother	Faecal
Nem	Male	09/06/02	2y 11m	Juvenile	Assiniboine	Mar/MoJ	Mother	Faecal
Jor	Male	04/04/03	2y 1m	Juvenile	Assiniboine	Mar/Jal	Mother	Faecal
Len	Female	07/10/03	1y 7m	Infant	Assiniboine	Mar/Jet	Mother	Faecal
Kob	Male	11/10/03	1y 7m	Infant	Assiniboine	Mar/JoJ	Mother	Faecal
Nay	Female	20/10/03	1y 7m	Infant	Assiniboine	Mar/MoJ	Mother	Faecal
Nag	Male	04/10/04	0y 7m	Infant	Assiniboine	Mar/MoJ	Mother	N/A *
Jam	Female	28/11/04	0y 7m	Infant	Assiniboine	Mar/Jal	Mother	N/A *
Jag	Male	22/03/05	0y 7m	Infant	Assiniboine	Mar/JoJ	Mother	N/A *

Table 2.5 – *Details of lion-tailed macaque subjects at San Diego Wild Animal Park (WAP). Individuals were housed in a group of 12 except ESH* who was housed on his own in close proximity to the rest of the group and was vasectomised. Samples from Esh* were only used for assay validation (Chapter 3).*

Subjects	Sex	Date of birth	Age class	Place of birth	Sire/Dam	Rearing	Samples collected
Gin	Female	16/12/77	Adult	San Diego zoo	Ami/Gin	Mother	Urine/Faecal
Esh*	Male	26/02/84	Adult	San Diego zoo	?/Pol	Mother	Urine/Faecal
Ada	Male	17/08/84	Adult	Woodland	Lit Joe/Lil	Mother	Urine/Faecal
Ste	Female	21/03/85	Adult	San Diego zoo	Pep/Lin	Mother	Urine/Faecal
Gra	Female	16/05/87	Adult	Royal zoo, Monaco	Pep/Cel	Mother	Urine/Faecal
Cha	Female	20/12/89	Adult	San Diego WAP	Tuf/Lin	Mother	Urine/Faecal
Ant	Female	12/02/92	Adult	San Diego WAP	Tul/Gin	Mother	Urine/Faecal
Eli	Female	05/09/93	Adult	San Diego WAP	Tul/Ste	Mother	Urine/Faecal
Cel	Female	02/07/98	Adult	San Diego WAP	Pie/Gra	Mother	Urine/Faecal

2.1.1 Chester Zoo housing and husbandry

The lion-tailed macaques had access to both an indoor (1092 m^3) and outdoor area (792.54 m^2) connected by three tunnels of 2.8 m in length (appendix 1a). The indoor enclosure had wood bark deep litter as a floor substrate and branches (approximately 18), ropes (approximately 15), poles (approximately 7) and platforms (approximately 9) were provided for climbing on. The outdoor area was a naturalistic island with grass covering surrounded by a water moat, with numerous shrubs (approximately 50), trees (approximately 20), poles (approximately 25), platforms (approximately 5) and ropes (approximately 10), and a waterfall.

Animals had access to the outside area at all times with the exception of cleaning times (which were typically 30 minutes long and occurred before 1000 hours daily) and enclosure maintenance. The animals were scatter fed on various seeds, nuts and primate pellet (approximately 600g) in the morning and were provided with approximately 2kg of a variety of fruits and vegetables (apple, orange, pear, grapes, banana, lettuce, bread, broccoli, celery, carrot, spinach, kiwi, pineapple, mango, radish and tomato) at random twice daily. Food enrichment was provided approximately twice per week in the form of browse, live insects, egg, apple custard, flowers, jam, honey, rice and ice blocks. Water was available *ad libitum*.

2.1.2 Bristol Zoo housing and husbandry

The lion-tailed macaques had access to both an indoor area split into two areas (134.20 m^3) and outdoor area again split into two (approximately 457.38 m^3) connected by four tunnels varying in length (appendix 1b). The indoor enclosures had wood wool as a floor substrate and branches (approximately 20), ropes (approximately 12) and platforms (approximately 10) were provided for climbing on. The outdoor area was made of two wire mesh enclosures with branches (approximately 20), tyres (approximately 2), ropes (approximately 6) and platforms (approximately 4) to climb on. The outside floor had a mesh lining and straw substrate was sometimes provided.

Animals had access to the outside area at all times with the exception of cleaning times (before 1100 hours daily) and enclosure maintenance. The animals were scatter fed on primate pellet (approximately 400g in total) in the morning. In the afternoon they were provided with approximately 2kg in total of a variety of fruits and vegetables (apple, grapes, mango, pineapple, papaya, melon, pepper, green beans, carrot, parsnip, pear, kiwi, plum, leek, celery, sweet potato, tomato, chicory, aubergine and broccoli). Food enrichment was provided approximately twice a week in the form of browse, eggs, cheese and yoghurts. Water was available *ad libitum*.

2.1.3 Assiniboine Park Zoo housing and husbandry

The lion-tailed macaques had access to both an indoor enclosure (19.2 m^3, split into three areas) and outdoor enclosure (360m^3), connected by a 1.8 m tunnel at floor level (appendix 1c). The indoor enclosures had platforms (approximately 9) and a few objects (approximately 5) to climb on. The outdoor area was a wire mesh enclosure with concrete floor and an area of grass. There were branches (approximately 12), ropes (approximately 3), barrels (approximately 3) and ladders (approximately 3) to climb on and regular enrichment was provided.

Access was given to the outside area at all times with the exception of cleaning times (before 1030 hours daily) and enclosure maintenance. The animals were scatter fed on cereals, raisins, crackers, marshmallows, dried fruit and popcorn (approximately 200g in total) and were provided with a variety of fruits, vegetables (banana, apple, orange, grapes, pear, strawberries, blueberries, peanuts, potato, sweet potato, beetroot, celery, broccoli, cauliflower, pepper, eggs, bread) and alfalfa (approximately 2kg) in the morning. In the early afternoon they were given a small fruit and vegetable feed (approximately 200g in total) and then a larger fruit and vegetable feed later in the afternoon (approximately 6kg in total).

Food enrichment in the form of browse, yoghurts, oatmeal cake, mealworms and puzzle boxes was provided approximately twice a week. Water was available *ad libitum*.

2.1.4 San Diego Wild Animal Park housing and husbandry

The lion-tailed macaques had access to a large outdoor corral enclosure (1590 m^2). The corral was furnished with three sheltered platforms and six trees, two of which were fallen. The floor substrate was grass and there were areas with shrubbery and a pond. Animals had access to the corral area at all times with the exception of sample collection times (between 1000 and 1400 hours daily) and enclosure maintenance. When the above took place animals were moved into a holding area that was connected to the corral by two tunnels at floor level (appendix 1d). This area consisted of three mesh cages (21.39 m^3, 145 m^3, and 29.25 m^3), two with concrete floor and one with dirt substrate floor. Elevated platforms (two) and two branches furnished one of the enclosures while the other two had three branches in each.

The animals were fed each morning approximately 2kg of a variety (the selection differed by day) of fruit and vegetables (greens, carrot, sweet potato, banana, pepper, corn, apple, orange, grapes, melon). Nuts and mealworms were given as a reward when urine and faecal samples were collected.

Food enrichment in the form of live crickets, mealworms and whole watermelon were provided approximately four times per week. Water and primate pellet was available ad libitum.

2.2 Behavioural observations and proximity recording

2.2.1 Behavioural observations

Behavioural data was collected from three groups of lion-tailed macaques housed at Chester (16 weeks), Bristol (12 weeks), and Assiniboine Park Zoo (12 weeks). Only three groups were studied due to a limited number of collections housing suitable social groups of lion-tailed macaques (breeding groups with which both samples and behaviour data could be collected from). The same behavioural observation schedule was used in all zoos. Behavioural data on interactions between individuals within the group were recorded using a range of behaviour sampling methods (Altmann, 1974). Instantaneous sampling was carried out every 30 seconds to provide a general overview of behaviour. One-Zero sampling was carried out during the 30 second time period assessing the occurrence of more frequent behaviours. Finally behaviours that were rarer in occurrence were recorded continuously by recording all of their occurrences. Focal animal observations lasting five minutes were carried out per individual per observation session. Three observation sessions were carried out per day at constant times throughout the study, in the morning (0900 hours) midday (1200 hours) and in the afternoon (1500 hours). These were carried out twice weekly, allowing each individual to be observed for 30 minutes per week. Data were collected on all individuals in the group when the study commenced (data were not collected on individuals born into the group during the study period) to give an accurate picture of the social relationships.

The order of focal animal samples in each observation period was randomised but at the same time it was ensured that each animal received equal amounts of observation for each time slot overall.

The ethogram of behaviours (adapted from Johnson, 1985; Skinner & Lockard, 1979) that were recorded by the focal animal sampling can be seen in table 2.6. This table also shows the sampling method that was used for

each behaviour. The data were recorded using the Noldus Observer Software Version 5 on a Psion "work about".

Table 2.6 – *The ethogram of behaviours that were recorded (adapted from Johnson, 1985; Skinner & Lockard, 1979) and the sampling method used. * Indicates that modifiers of the behaviour were recorded, for example, the animal that the particular behaviour was directed towards and/or whether the animal gave or received the behaviour.*

Behavioural Category	Definition
Instantaneous	
Inactive	*Any stationary behaviour that occurred in the absence of all other behaviours including sit, upright, stand, lie down, sleep, sprawl, hang.*
Locomotion	*Movement with the purpose of getting to another area of the enclosure. Any mutually exclusive form of movement that occurred in the absence of other behaviours including walk, run, climb, leap, hop, swing.*
Social	*Any form of social behaviour observed on the instantaneous sample.*
Foraging	*Any behaviour relating to searching for food, including hunting and food manipulation away from the mouth.*
Feeding	*Any behaviour related to feeding including food manipulation near the mouth.*
Drinking	*When individual was actively drinking water.*
Clinging	*When infant was the focal animal and was clinging either ventrally or was on the back of another individual.*
Out of sight	*When the observer could not see the individual on the instantaneous sample and therefore its activity could not be recorded.*

One-Zero	
Auto-grooming	*Grooming of self by manipulation of the fur with fingers or mouth.*
Allo-grooming	*Grooming of another individual by manipulation of the fur with fingers or mouth.*
Self object play	*Any instance of play behaviour towards an object that did not appear to be directed at another individual or that did not appear to have the aim of initiating social play. Also any behaviour of an investigatory nature towards an object (for example stick) or other animal (commonly mouse/rat). Behaviours included look, watch, sniff, lick, manipulate, lift, rub, and wrestle.*
Social play *	*Any instance of social play behaviour, including play initiation, bouncing gait, chase, play wrestle, play bite, and play termination.*
Exploratory	*Any behaviour of an investigatory or playful nature not towards objects. This behaviour was commonly displayed by infants.*
Infant related	*Any contact behaviour related to an infant involving the infants' dam, sire or other group members. Behaviours included root, nipple contact, cling, gather, collect, restrain, pat, arms around, pick up, carry, turn, put down, examine, support, withdraw.*
Threat facial display*	*Facial display of a threatening nature including staring open mouth, forward bared teeth, crouch face threat, tense mouth threat and head toss.*
Appeasement facial display *	*Facial display for appeasement including grimace, relaxed open mouth face (grin) and pout face.*
Mixed-context facial display *	*Facial display with a mixed context including raise brow/flatten ears, purse lips, lipsmack and yawn.*
Growl Vocalisation	*A growling vocalisation, which appeared to be aggressive in nature and was usually directed towards keepers or other animals.*
Squeal vocalisation	*A squeal vocalisation usually seen in response to threat or aggression.*
Cluck vocalisation	*A cluck vocalisation usually made by infants when seeking maternal protection.*

Whine vocalisation	*A whine vocalisation usually seen prior to and during feeding and foraging.*
Grunt vocalisation	*A grunt vocalisation - females were seen to grunt when a male dismounts after mating. Males may grunt during aggressive acts.*
Continuous (All Occurrences)	
Sexual inspection *	*Male pulling or brushing aside females' tail and then visually inspecting her vulva and anus. Male used his finger to aid inspection and may also sniff area. Females may also inspect other females in a similar manner.*
Mount *	*Any instance of mounting by males or females. Males mounted females by wrapping their feet around her ankles and their hands grasp the midsection of the back. Females mounted other females or males however they did not wrap their feet around the individuals ankles and their hands were further posterior.*
Mating *	*Mounting of female when in oestrus by male, accompanied by rhythmic pelvic thrusting.*

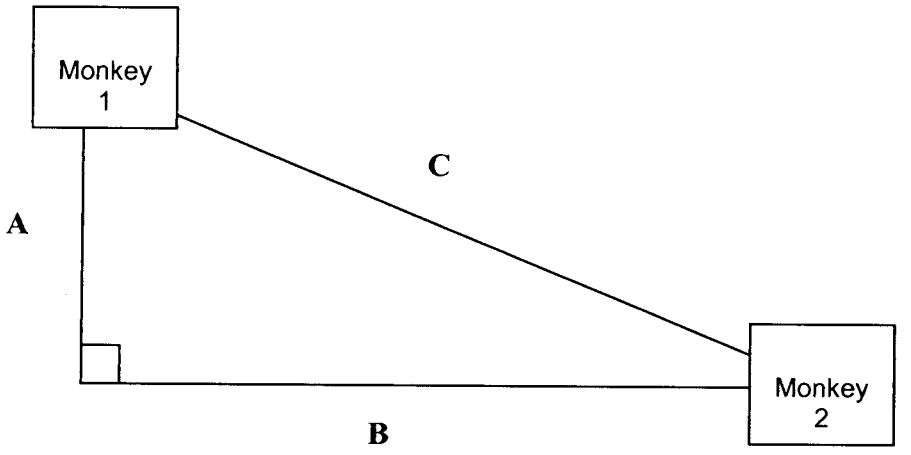
2.2.2 Measure of spatial proximity

Spatial proximity of individuals in the group were recorded to provide an index of social relationships. Each enclosure was divided into virtual, 3-dimensional spaces (grids, ranging from 0.3 x 1.3m to 22.2 x 18.7m in size). Spatial proximity was recorded by scan sampling from left to right of the enclosure every 5 minutes (at the start and end of each focal animal sample period [therefore Chester = 24, Bristol = 21 and Assiniboine = 51 scans per day]). The location of each individual (its grid reference) was recorded from an enclosure plan. The indoor and outdoor enclosure plans for each of the three zoos and their grid references are shown in appendix 1. Whether or not the focal animal was within "arms-reach" of any other individuals (total number and identification) was also recorded by instantaneous sampling every 30 seconds during each focal animal observation. This was recorded by hand on a record sheet (appendix 2).

2.2.3 Analysis of spatial distances

Data on the location of subjects were collected in the form of virtual 3-dimensional grid references for each zoo enclosure. This was subsequently converted to X, Y and Z co-ordinates (distance from the origin in metres) as shown in appendix 3. Pythagoras' Theorem (figure 2.1) was then used to calculate distances between individuals using a specifically designed spreadsheet (Microsoft Excel 2000). The spreadsheet was adapted to accept coded grid references unique to each zoo enclosure. Raw data were entered into the computer and a spreadsheet was programmed to calculate distances between individuals in each group.

Figure 2.1 – *Pythagoras' Theorem states that the square of the hypotenuse of a right-angled triangle is equal to the sum of the squares on the other two sides ($C^2 = A^2 + B^2$). Parameters A and B were determined using X, Y and Z coordinates, the program then calculated C (the distance between the monkeys).*



2.3 Non-invasive urine and faecal sample collection

Samples were collected for a minimum of 12 weeks from each of the three groups of lion-tailed macaques, housed at Chester, Bristol and Assiniboine Park zoo. Additional samples were also collected from San Diego Wild Animal Park for a seven day period. Faecal and urine samples were collected from all animals opportunistically except in the case of San Diego Wild Animal Park where the animals had been previously trained (refer to section 2.3.4). Samples that may have been contaminated by other faeces or urine were not used in analysis. The exact method of sample collection varied between institutions (see below).

All sample collection procedures were approved by the ethical review and research committees at each institution. At no point did the researcher enter the cage with a subject. Disposable gloves and a lab coat were worn at all times for self-protection.

Samples were usually collected straight from the concrete or enclosure floor onto which they were deposited however when possible urine samples were collected in aluminium trays held underneath the animal when in an overhead

tunnel (in the case of Chester Zoo subjects). Urine samples were pipetted using disposable pipettes and transferred to plastic eppendorf tubes and faecal samples were collected using disposable tongue depressors and transferred into plastic sample bottles labelled with the date, time and animal name. Samples were collected within four hours of production (as discussed below) and stored at -20°C.

2.3.1 Chester Zoo sample collection

Samples were collected throughout the day (between 0700 hours and 1700 hours) when the animal was in the overhead tunnel (see section 2.1.1 and appendix 1a) or (in the case of faeces) if the sample was deposited in a noticeable area collected when keepers cleaned the enclosure. Samples were collected within one hour of production and frozen.

2.3.2 Bristol Zoo sample collection

Samples were observed being produced in the enclosure between 0730 and 1100 hours and were collected when keepers cleaned the enclosure. Samples were collected within two hours of production and frozen.

2.3.3 Assiniboine Park Zoo sample collection

Faecal markers, non-toxic glitter and food dye (Lord, 2001) in different colours were added to prunes (approximately 0.2g of glitter per 8.4g of prune) and hand fed individually to each animal by two people (the researcher and primate keeper) the afternoon on the day before sample collection. A different colour was assigned to each individual and the next day any fresh samples identified by the faecal marker were collected before 1000 hours. Samples were probably collected and frozen within a maximum of four hours since production as animals awoke at approximately 0600 hours.

2.3.4 San Diego Wild Animal Park sample collection

The subjects at San Diego Wild Animal Park were trained approximately five years prior to the study (not by the current researcher) to enter a series of tunnels and produce a faecal and urine sample by positive reinforcement (a food reward – see appendix 1d). Samples were collected immediately after production and this took place between 1000 and 1400 hours.

2.4 Extraction and ELISA procedure

The methods below were scientifically validated. The validation is described in more detail in chapter three.

2.4.1 Faecal extraction procedure

Faecal samples were extracted before analysis. Samples were defrosted and any foreign bodies removed before approximately 1g of faeces was weighed out. Samples were then dried at 40°C for six hours (table 2.7) over two days and refrigerated overnight. On occasion samples were not dry after six hours due to water content in the sample (water content in faeces can range between 30-75% of faecal mass and vary from sample to sample within an individual or between individuals) (Ziegler *et al.*, 1996; Ziegler & Wittwer, 2005). If samples were not dry after six hours they were left in the oven for a further three hours maximum. A pestle and mortar were used to crush the faecal matter into a powder and solvent was used to clean the pestle and mortar in between samples. Faecal powder (0.2g) was weighed out and 3ml of 95% methanol added. Samples were then vortexed for one minute before being placed on a shaker for three hours. The shaking time of three hours was chosen as it gave the same results as samples that were shaken for 6 hours and was less time consuming.

Following removal from the shaker samples were centrifuged for 15 minutes at 2000 rpm and the solvent decanted off. Samples were then dried at 35°C under Nitrogen and reconstituted in 1 ml EIA Phosphate buffer solution (EIA

PBS) (0.1M PBS:5.42g $NaH_2PO_4H_20$, 8.66g Na_2PO_4 [Anhydrous], 8.7g NaCl, 1.0g BSA [RIA grade Albumin Bovine] 1L dh_20, pH 7.0). Samples were vortexed for three minutes and stored at -20°C until analysis. Before analysis both urine and faeces samples were defrosted and centrifuged for one minute, and the supernatant (500 µl) was separated from any particles into a clean micro-centrifuge tube.

Table 2.7 – *Table showing the results of an experiment to discover the number of hours of oven drying required at 40°C for faecal samples. Samples (n=4) had 1g removed and dried in the oven. Samples were classed as sufficiently dry when their weight remained constant.*

Sample number	Weight (g) of faecal sample after time dried at 40°C (hr)						
	0	1	2	3	4	5	6
1	1.0	0.56	0.41	0.40	0.39	0.36	0.36
2	1.0	0.48	0.42	0.39	0.39	0.33	0.33
3	1.0	0.46	0.37	0.33	0.32	0.39	0.39
4	1.0	0.52	0.35	0.37	0.36	0.39	0.39

2.4.2 Cortisol ELISA procedure

Anti-cortisol antibodies and horseradish peroxide conjugated cortisol were obtained from Coralie Munroe (University of California, Davis, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Assays were performed in 96-well microtiter plates (Maxisorp, NUNC ™) that were precoated the previous day with antibody (R4866, raised against a steroid bovine albumin in rabbit (Munro & Stabenfeldt, 1985)). Due to unforeseen circumstances the first batch of cortisol antibody and the steroid conjugate, cortisol horseradish peroxide (Hrp), ran out and so part way through the analysis a second batch of antibody and Hrp was used. The specificity part of the immunological validation was carried out for each batch of antibody (see chapter 3). For this reason all relevant dilutions will be given twice – once for the first batch and once for the second respectively.

The antibody was diluted to 1:15,000 (batch 1) or 1:12,000 (batch 2) in 0.05M coating buffer (1.59g Na_2CO_3, 2.93g $NaHCO_3$, 1L dh_20, pH 9.6). Ziegler et al (1995) have reported this antibody to have cross reactivities of 96% with prenisolone, 66% with prednisone, 60% with cortisone, 2.5% with corticosterone and < 1% with various other steroids. The antibody dilution was added to 94 wells (50µl/well), in the remaining two wells 50µl dh_20 was added to control for non-antibody binding and the plate was then sealed and incubated overnight at 4°C.

The next day the plates were emptied of excess antibody and washed six times, three times in each direction to minimise potential drift (10:1 EIA wash solution: 87.7g NaCl [1.5M], 5 ml Tween 20 [0.5%], 1L dh_20, 350µl/well). EIA PBS (50µl) was pipetted into all the wells followed by 50µl standards (diluted in dh_20 for urine and EIA PBS for faeces [Sigma; n=10, 1.95 to 1000pg]). Samples were diluted in dh_20 for urine (1:128) and EIA PBS for faeces (1:4 to 1:32) in duplicate in the appropriate wells. Finally 50µl of cortisol (Hrp) diluted to 1:30,000 (batch 1) 1:22,000 (batch 2) in PBS was added. The plates were sealed and incubated for 2.5-3 hours at room temperature in the dark.

The plates were washed six times as before and 100µl substrate solution (12.5ml EIA citrate buffer [0.05m, pH 4.0:9.61g Citric Acid (Anhydrous), 1l dh_20], 125 µl EIA ABTS [40Mm, 2,2'-AZINO-bis (3–ETHYLBENZTHMOLINE–6–SULFONIC ACID) Diammonium salt], 0.329g ABTS, 15ml dh_20, pH to 6.0; 40µl EIA H_20_2 [2.0%, 0.5M, 500µl H_20_2 (30%) 8M, 7.5ml dh_20]) was added to all wells. The plates were then shaken and read using the software Revelation version 4.22 on a microplate reader (Dynatech MR700) until the optical density at 405 nm for the control (B_o) wells containing zero hormone measured 1.0.

All samples were assayed in duplicate and any sample with a CV higher than 10% was repeated. Any sample that fell outside of the parallel portion of the

standard curve (10-90% binding) was diluted at a different dilution and re-assayed to ensure maximum sensitivity of each measurement.

2.4.3 Creatinine assay

The hormone concentration for each urine sample was corrected for creatinine concentration using a modified Jaffe end-point assay (Burtis and Ashwood, 2001) to control for varying urine concentrations. All urinary cortisol concentrations were expressed relative to creatinine (cort/ml/mg cr).

Creatinine assays were carried out using 96-well non-bonding microtiter plates (Maxisorp, NUNC ™). Two separate urine pools were made up (see chapter 3 section 3.2.3.1 for more information). Standards (Sigma, n=4, 0.75µg to 6µg per 500µl) and samples (diluted at 1:32 for the first pool and 1:128 for the second pool) were diluted in dh_20 and 200µl pipetted into the appropriate wells in duplicate. Control wells contained 200µl of dh_20 as an indicator of non-specific binding. An equal mix of picric acid and NaOH was added to all but the control wells (100µl/well). Plates were shaken for 1-2 minutes. After 10-15 minutes the plates were read using the software Revelation version 4.22 on a microplate reader (Dynatech MR700) when the optical density at 490nm of the top standard (6µg per 500µl) measured 1.5-1.7.

2.4.4 Extraction efficiency

The extraction efficiency of faecal samples was carried out by Dr Alison Forhead and Dr Joanna Setchell at University of Cambridge, UK. Previously oven dried faecal samples (n=5, 0.2g) were spiked in duplicate (n=10, 3 were misplaced resulting in n=7) with 600µl of tritiated hormone (Amersham, Buckinghamshire, UK) to achieve an approximate count per minute (cpm) of 3000. Tritiated hormone (600µl) was also added to four control tubes with no faeces. Samples were extracted following the process described in this chapter, section 2.4.1. Scintillation cocktail (4ml, Optiphase, 'hi-safe 2',

PerkinElmer, Wellesley, Massachusetts, USA) was then added to the samples and the controls. Samples and controls were counted using a 1216 RackBeta counter (LKB Wallac) and the percentage of procedural loss during extraction calculated (cpm of control samples/cpm of extracted samples).

2.5 Data analysis

Data were organised using Microsoft Excel 2003 and analysed using SPSS statistical package version 12. Behaviours were proportioned for time spent in sight. For example, if an individual was observed allogrooming on 1 in 10 occasions in a focal animal observation and was in sight of the observer for only 4 of the 10 occasions, then the individual was observed allogrooming for 25% of the time. Observations where the focal animal was out of sight for more than 80% of the time were not included in the analysis. Lion-tailed macaque subjects were divided into age groups (section 2.1, table 2.1) for analysis based on previous research (Green & Minkowski, 1977; Lindburg, 2001; Rowe, 1996).

2.5.1 Statistical analysis

Parametric tests were used over non-parametric tests as they are more powerful. Furthermore non-parametric tests may not detect differences or relationships in data even when they actually exist (Pallant, 2005). Parametric statistics have a range of assumptions, the primary one being that the data is normally distributed. If data is not normally distributed then it can be mathematically modified to reduce the effect of outliers or extreme values (Pallant, 2005). Data for this thesis was not normally distributed so two transformations were performed to achieve normality, logarithmic and arcsine. Arcsine transformation was used on data in this thesis that was proportioned for example behavioural data presented as a proportion of time spent in sight. All other data were logarithmically transformed.