Linking Welfare and Quality of Scientific Output in Cynomolgus Macaques (*Macaca fascicularis*) used for Regulatory Toxicology

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Linking Welfare and Quality of Scientific Output in Cynomolgus Macaques (*Macaca fascicularis*) used for Regulatory Toxicology

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"The proper utilization of our intelligence and knowledge is to effect changes from within to develop a good heart."

Dalai Lama.

"You must be the change you want to see in the world."

Mahatma Gandhi.

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Chapter 4 Refinement of housing and husbandry for cynomolgus macaques (*Macaca fascicularis*) and the effects on biological data

Acknowledgments

"Knowledge is in the end based on acknowledgement."

Ludwig Wittgenstein.

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>
> *~ Christopher Robin to Winnie- The-Pooh*
> A.A. Milne (1936).

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Presentations

Buchanan-Smith HM, Kelly J & Tasker L 2011 Good welfare – good scientific output: Refining husbandry and procedures for primates in the laboratory. Invited presentation at the 11th BfR Consumer Protection Forum "Welfare of Laboratory Animals - Role of Refinement?" Berlin, Germany, December.

Tasker L, Kelly J & Buchanan-Smith HM 2011 Good welfare – good science: Refining toxicological procedures for cynomolgus macaques (*Macaca fascicularis*) through enhanced socialisation with care-staff. Oral presentation in a session entitled "*Animal Welfare for Refinement and High Quality Science*" at 8th World Congress on Alternatives and Animal Used in the Life Sciences, Montreal, Canada, August.

Tasker L, Allison R, Owen S, Kelly J & Buchanan-Smith HM 2011 Refining husbandry and handling practices for laboratory-housed primates. Invited oral presentation at LASA/UFAW 3Rs section meeting. London, UK, June.

Tasker L, Kelly J & Buchanan-Smith HM 2010 Assessment of welfare: cynomolgus monkeys. Invited presentation on EUPRIM-Net course on General Primate Biology for Animal Caretakers and Technical Personnel, Defence Science and Technology Laboratory (DSTL), Porton Down, UK, September.

Burnett J, Tasker L, Brook J, Price C & Hanson-Williams K 2010 Refining dried blood spot techniques for toxicokinetic data derived from cynomolgus macaques. Poster presentation in a session entitled "Regulatory and Risk Assessment" at XII International Congress of Toxicology organised by International Union of Toxicology, Barcelona, Spain, July.

Tasker L, Kelly J & Buchanan-Smith HM 2010 Linking welfare and science: Ten years of welfare-positive housing and husbandry changes and the effects on biological data of cynomolgus macaques used for regulatory toxicology. Oral presentation in a platform session entitled "*Non-human primates – value of models and progress with the 3Rs*" at "New Paradigms in Laboratory Animal Science" triennial meeting of Federation of European Laboratory Animal Sciences Association Helsinki, Finland, June.

Bodey A & Tasker L 2010 Future challenges - Training primates to cooperate with procedures in regulatory toxicology. Oral presentation in a workshop entitled "*Beyond Housing…adding value with the 3Rs*" at "New Paradigms in Laboratory Animal Science" triennial meeting of Federation of European Laboratory Animal Sciences Association Helsinki, Finland, June.

Tasker L, Burnett J, Brook J, Price C & Hanson-Williams K 2010 Refinement of blood sampling techniques for the non-human primate to provide dried blood spot samples for generation of toxicokinetic data. Poster presentation at "New Paradigms in Laboratory Animal Science" triennial meeting of Federation of European Laboratory Animal Sciences Association Helsinki, Finland, June.

Buchanan-Smith HM & Tasker L 2010 Linking welfare and quality of science in non-human primates. Invited Presentation in the NC3Rs symposium at the Institute for Animal Technology Congress, April.

Buchanan-Smith HM, Bowell V, Tasker L, Kelly J & Schnell C 2010 How humans influence animal welfare and quality of science. Invited presentation at the Enrichment Extravaganza, Boston, USA, April.

Tasker L 2009 Socialisation. Invited presentation on a Institute of Animal Technology (IAT) continuing professional development course entitled Primate Training, University of Stirling, Scotland, October.

Tasker L & Buchanan-Smith HM 2009 Training primates to refine husbandry and scientific procedures. Poster presentation at the Laboratory Animal Science Association Winter meeting, UK, November.

Tasker L, Kelly J, Sutcliffe S & Buchanan-Smith HM 2009 The effects of an enhanced socialisation programme on behaviour, welfare and cardiac responses of newly acquired cynomolgus macaques (*Macaca fascicularis*) during six-week acclimatisation period. Oral presentation in a symposium entitled "Primate Stress – Causes, Responses and Consequences." Organised by Primate Society of Great Britain UK, London, UK, December.

Tasker L, Kelly J & Buchanan-Smith HM 2009 Integrating measures of behaviour, physical health and physiology to produce an overall assessment of welfare in the cynomolgus macaque (*Macaca fascicularis*). Poster presentation in a symposium entitled "Primate Stress – Causes, Responses and Consequences." Organised by Primate Society of Great Britain UK, London, UK, December.

Tasker L, Kelly J & Buchanan-Smith HM 2009 *Good welfare, good science:* Training macaques for scientific procedures in regulatory toxicology. Invited presentation in a symposium entitled "All animals wild and tame, part II – training of laboratory animals used for regulated procedures". Organised by the Laboratory Animals Veterinary Association, Manchester, UK, September.

Tasker L, Kelly J & Buchanan-Smith HM 2009 *Good welfare, good science:* Training macaques for scientific procedures in regulatory toxicology. Invited presentation in a symposium organised by Vet Nurses in Research Association, Annual Congress, UK, Manchester, UK, September.

Tasker L, Kelly J & Buchanan-Smith HM 2009 Assessing the welfare of cynomolgus macaques (*Macaca fascicularis*) for evaluating planned Refinements. Poster presentation given at the NC3Rs parliamentary event entitled "The Three R's Today" London, UK, March.

Tasker L & Buchanan-Smith HM 2009 The effects of Refinements on experimental outcomes in macaques. Invited presentation in a symposium entitled "Enrichment, Animals and Experimental Outcomes" organised by the Universities Federation for Animal Welfare /Laboratory Animal Science Association, Stevenage, UK, February.

Kelly J, Tasker L & Buchanan-Smith HM 2009 Assessing the welfare of primates for evaluating Refinements in regulatory toxicology. Poster presentation given at the European Parliamentary Poster Reception on the 3Rs hosted by the BioSciences Federation, Brussels, Belgium, February.

Tasker L 2008 *Macaca fascicularis* – Handling and Animal Welfare. Invited presentation on a Institute of Animal Technology (IAT) continuing professional development course entitled Primate Training, University of Stirling, Scotland, October.

Tasker L, Smith T, Kelly J & Buchanan-Smith HM 2008 A tool to assess the welfare of cynomolgus macaques (*Macaca fascicularis*) for use in evaluating planned refinements in the laboratory. Poster presentation given at the International Primatological Society Conference, Edinburgh, UK, August.

Buchanan-Smith HM, Badihi I, Tasker L, Bassett L, McKinley J & Morris K 2008 *Assessment of zoo animal welfare: Lessons from laboratories. Oral presentation in a symposium entitled "* Measuring Zoo Animal Welfare: The Science of Animal Well-being, Combining Approaches and Overcoming Challenges" organised by the Chicago Zoological Society, Brookfield Zoo, Brookfield, Illinois, USA, May.

Tasker L & Buchanan-Smith HM 2008 Identifying behavioural indicators of welfare for laboratory-housed cynomolgus macaques (*Macaca fascicularis*). Poster presentation given at the Scottish Conference on Animal Behaviour, Stirling, UK, April.

Abstract

> *"We must first distinguish* direct *and* contingent *inhumanity. By the former, we mean the infliction of distress as an unavoidable consequence of the procedure employed, as such, even if it is conducted with perfect efficiency and completely freed of operations irrelevant to the object in view.*
>
> *By contingent inhumanity, on the other hand, we mean the infliction of distress as an incidental and inadvertent by-product of the use of the procedure, which is not necessary for its success. In fact contingent inhumanity is almost always detrimental to the object of the experiment, since it introduces psychosomatic disturbance likely to confuse almost any biological investigation."*
>
> Russell & Burch (1959), p 54.

*Cynomolgus macaques (*Macaca fascicularis*) are the most commonly used non-human primate for research and testing in Europe. Their principal use is in preclinical safety testing of new pharmaceuticals to assess risk of adverse effects, as indicated by changes in a core battery of physiological measures before human exposure. Regulatory studies are strictly controlled through legislation and codes of practices underpinned by the principles of humane science, the 3Rs; Replacement, Reduction and Refinement. Despite the link between good welfare and good science now universally made in codes of practice, legislation and the literature, there are few studies aimed at systematically examining the link and almost no quantitative data from cynomolgus macaques used for toxicology. The main aim of this thesis was to examine the link between Refinement, animal welfare and scientific output for this important animal model, piggy-backing on regulatory studies conducted by a large contract research organisation.*

In the laboratory, animal welfare is formally considered in terms of Refinement which has evolved to include both the reduction of negative welfare states and the proactive enhancement of positive welfare over the animal's lifetime. A multidisciplinary approach to welfare assessment including measures of behaviour, physiology and physical health, and which built upon current unit procedures was undertaken to produce an overall assessment of welfare in cynomolgus macaques. Macaque facial expressions, vocalisations, activity and position in the home cage, body weight change, body condition and alopecia scores were found to be reliable indicators of welfare state and would be most feasible for care staff to monitor.

The concept of quality of scientific output was defined in relation to toxicological findings and includes sensitivity, reliability and repeatability of individual measures in the core battery (e.g. heart rate, blood pressure, haematology, clinical chemistry and organ weights). The link between welfare and quality of scientific output was then systematically explored with Refinements to macaque use in regulatory studies. The first, a data mining study, undertaken to quantify the effects on biological data recorded from cynomolgus macaques, used in regulatory studies over an eight-year period as the CASE sponsor transitioned from single to permanent group housing, found the effects to be highly variable on individual parameters in the core battery and in some instances welfare-positive effects of group housing were confounded by concurrent changes in standard operating procedures. A further study of planned Refinements to macaque-care staff interaction through enhanced socialisation was found to help animals cope better with husbandry and scientific procedures and enhance quality of cardiovascular measures recorded at baseline. In light of these findings a number of recommendations are made including a framework of terms useful for measuring quality of scientific output, a welfare assessment framework and Refinements to husbandry and scientific procedures for cynomolgus macaques used in regulatory toxicology. Because of their capacity to suffer it is both ethically and scientifically important that macaque welfare is maximised and their use results in valid and reliable experimental outcomes informing on the safety and efficacy of new pharmaceuticals prior to human exposure.

Preface

I. Taxonomy

Order	Primate
Suborder	Haplorrhini
Infraorder	Catarrhini
Superfamily	Cercopithecoidea (Old World monkeys)
Family	Cercopithecidae (i.e. macaques)
Subfamily	Cercopithecinae
Genus Sp.	*Macaca fascicularis*
Subspecies (10)	*M.f. atricepts; M.f. aurea; M.f. condorensis; M.f. fascicularis; M.f. fusca; M.f. karimondjawae; M.f. lasiae; M.f. philippinensis; M.f. tua; M.f. umbrosa* Likely Mauritian subspecies: *M.f. fascicularis*
Common names	Long-tailed, crab-eating, kra, cynomolgus

From Groves (2001).

II. Location and distribution of wild populations

The majority is located in mainland Southeast Asia. Their range extends from Southeastern most part of Bangladesh, through Myanmar, and Southern two-thirds of Thailand and off shore islands; Malay Peninsula, Sumatra, Bangka, Java, Bali, Nusatenggara to Timor, Borneo, Bawean, throughout Cambodia, Southeastern tip of Laos and through Southern Vietnam; into Philippines, into Northern coast of Sumatra, Southern Indian Nicobar islands and introduced to islands of Sulawesi, West Papua, Mauritius, Hong Kong and Palau (Groves 2001; Gumert 2011).

III. Physical characteristics *of M. fascicularis*

Locomotion	Quadrupedal, terrestrial & arboreal. Hind limb dominated, posterior centre of gravity.
Physical characteristics	Grey-reddish brown pelage with lighter under parts. Pink face, light spot at corner of eyelid, crown hair directed backward and outwards sometime forming a small central crest. Tail length varies (range: 67-150% of crown-rump length; insular and peripheral populations differ). Sexually dimorphic: Males have cheek whiskers and moustache; females have beards; males bigger than females.

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The use of cynomolgus macaques (*Macaca fascicularis*) in regulatory toxicology

"Against this general background of fact and principle, we shall proceed to set the positive features of the subject: the removal of inhumanity by the three modes of Replacement, Reduction, and Refinement."

Russell & Burch (1959), p 66.

Abstract

*Cynomolgus macaques (*Macaca fascicularis*) are the most commonly used nonhuman primate for research and testing in Europe, the majority being used in regulatory toxicology studies undertaken to meet the legal requirements during drug development. They are an important* in vivo*, nonrodent animal model in toxicology for predicting the adverse effects of novel pharmaceuticals on a core battery of biological measures recorded at multiple time points, in-life and at end-of-life. Their use is strictly controlled through legislation and codes of practices outlining minimum standards for their housing, care and the conduct of procedures and underpinned by the principles of humane science, the 3Rs; Replacement, Reduction and Refinement. The design of a preclinical toxicology study depends on the intended therapeutic use of the test article in humans but it too is governed by international guidelines intended to harmonize approaches and reduce animal use (e.g. International Conference on Harmonization). Nevertheless the use of primates for research and testing is contentious; their complex cognitive, social and psychological needs make providing appropriate environments and caring for them over their lifetime with constraints of the laboratory and study requirements, a permanent challenge for breeders and research facilities. In the UK a utilitarian approach is adopted for dealing with ethical dilemmas involving the use of animals in research. Where the use of primates is indicated a harm-benefit assessment and the 3Rs principles should be applied so that they are only used when absolutely necessary, morally justified, and animal suffering is kept to a minimum. Because of their capacity to suffer it is critical that their welfare is maximised and their use results in valid and reliable experimental outcomes on the safety and efficacy of new pharmaceuticals prior to human exposure.*

1.1 Introduction

This Chapter gives an overview of the use of cynomolgus macaques for toxicology testing in Great Britain (GB). It narrates the legislative and regulatory requirements controlling research and testing, and also summarises the main ethical issues surrounding their use. The United Kingdom has a long and unique history of legislation controlling the use of animals in experiments. In 1876 the Cruelty to Animals Act was introduced, the first of its kind in the world to provide a legal basis for the control of 'painful experiments' on animals for specific purposes and under certain circumstances (Purchase *et al* 1998; Dolan 2000). It resulted from a growing concern about the inhumanity of some experiments from the general public, and many eminent scientists (including Darwin) petitioned the government for greater control (Dolan 2000; Feller 2009).

However, the many limitations with the Act meant that the numbers of animals rose steadily throughout the 21st century, reaching a peak in 1973 with 5.5 million animals per year being used for research purposes (Dolan 2000). Furthermore there was an increasing debate on the ethical justification for the use of animals in research (Dolan 2000). Surprisingly this Act was not repealed until the Animals (Scientific Procedures) Act in 1986. This enabling Act has the principles of humane science at its core, requiring scientists to Replace animals in experiments wherever possible, when this is not achievable the onus is on them to both Reduce the number of animals used and the suffering of individuals through Refinement. Furthermore its ethical framework is based upon the Utilitarian approach, weighing up the harms to animals used in experimental purposes vs. the potential benefits to humans and/or animals. In the case of toxicological research, animals are used to evaluate the adverse (toxic) effects of chemicals before they are used in humans:

> *"Since most of the research necessary for the safety evaluation of chemicals requires the killing of laboratory animals, toxicologists are faced with an ethical conflict between their professional duties and the interests of the animals. …… In recent years, toxicologists have become aware of their ethical responsibilities not only for the safety of the human population but also for the welfare of the animals."*
>
> Zbinden (1985), p137.

The next Sections give a current account on the use of cynomolgus macaques (*M. fascicularis*) for toxicology testing, the stringent legislative and regulatory controls that govern their use, care and housing, and the rationale and approach as models for evaluating the toxicity, safety and efficacy of new pharmaceuticals.

1.2 Legislative requirements

The use of non-human primates[1] in the United Kingdom (UK) is subject to provisions of The Animals (Scientific Procedures) Act (1986) and its associated Codes of Practice (Home Office 1986; 1989; 1995) which are underpinned by the principles of humane science (Russell & Burch 1959); the three Rs: ***Replacement, Reduction, Refinement. Replacement*** is concerned with the absolute or relative replacement of animals for scientific use; ***Reduction*** emphasizes the need to reduce to a minimum, the number of animals through good experimental design, sharing data and/or resources, or by using modern techniques to obtain more information from the same number of animals (thereby reducing future use of animals) and ***Refinement*** has been defined as:

> "*any approach which avoids or minimises the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved, and which enhances their well-being*"
>
> Buchanan-Smith *et al* (2005), p 379-380.

Refinement is concerned with the animals' lifetime experience (Figure 1.4) and fundamentally requires users of experimental animals to be able to recognise when their well-being is affected positively and negatively (Chapter 3) during breeding, in response to housing (Chapter 4), care, capture, restraint, and procedures (Chapter 5) planned or otherwise. The Act recognises that scientific procedures cause adverse effects on animals[2] and scientific work is strictly controlled through a system of licensing, including the place (designated establishment), the programme of work (project licence), and those conducting the procedures (personal licensees). The project licence requires applicants to estimate the likely severity of procedures performed, and is used to assess the 'harms' to the animal (Home Office 2000; Boyd Group and RSPCA 2004). Moreover

[1] Non-human primates are hereafter referred to as primates.
[2] Under the Animals (Scientific Procedures) Act (1986) any scientific procedure carried out on any living vertebrate animal, or one species of octopus *(Octopus vulgaris)*, which is likely to cause that animal pain, suffering, distress or lasting harm is considered a regulated procedure which requires a licence. The project licence covers the scientific programme of work and includes harm-benefit analysis, whilst personnel conducting the regulated procedure must also be licenced (personal licence).

individual licensees have personal responsibility for the welfare of animals on which they carry out a regulated procedure (Home Office 1986). The minimum environmental and husbandry requirements are set down in the Codes of Practice (Home Office 1989; 1995). From the outset of any intended scientific study the use of primates requires special justification under the Act, and permission is only granted if the Secretary of State is satisfied that no alternative test or animal can be used (Home Office 1986). The use of wild-caught primates is not permitted under the Act and no great apes have been used since the introduction of the 1986 Act[3].

At the time of writing, new European legislation; Directive 2010/63/EU The Protection of Animals used for Scientific Purposes (EU 2010) is currently being transposed into British law. The Animals (Scientific Procedures) Act (1986) is based upon the outgoing Directive: 86/609 EEC Protection of Animals used for Experimental and Other Scientific Purposes (EEC 1986) whose provisions will be replaced from 1st January 2013. The new Directive aims to reduce disparities between laws, regulations and administrative provisions of Member States with more stringent and transparent measures in areas of animal experimentation and differs from Directive 86/609 EEC with specific respect to the use of primates and more generally. The scientific justification for primate use and the type of research permitted will be restricted to that which is undertaken with a view to the avoidance, prevention, diagnosis or treatment of debilitating or potentially life-threatening clinical conditions in human beings. *In vivo* testing of substances and products remains a legislative requirement to provide appropriate safety and efficacy data prior to regulatory approval (EU 2010).

A fundamental difference with the new Directive is the greater emphasis on the lifetime experience (Figure 1.4) of animals and cumulative suffering potentially experienced within a given procedure. Authorisation of projects remains based upon the harm-benefit assessment in current use in the UK (Section 1.6.2) with additional requirements for researchers to retrospectively report the actual levels of pain, suffering, distress and lasting harm that was inflicted on animals. Procedures will be classified as 'non-recovery', 'mild', 'moderate', or 'severe' – a change to the somewhat confusing

[3] The Home Office will not grant a licence to use wild-caught primates unless there are exceptional scientific justifications for their use (Home Office 1986, 1989). A ban on the use of great apes was introduced in November 1997.

current system of severity bands for the project and limits for the procedures (Boyd Group and RSPCA 2004; APC 2003; 2009). The assignment of the severity category will be based upon the nature of pain, suffering, distress or lasting harm caused by (all elements of) the procedure, and its intensity, duration, frequency and multiplicity of techniques employed. It will also take into account any intervention or manipulation of an animal within a defined procedure and be based upon the most severe effects likely to be experienced by an individual animal after applying all appropriate Refinement techniques.

1.3 Source of cynomolgus macaques for research and testing

Historically, there has been a critical shortage[4] of captive bred monkeys, especially Old World (OWM) species for use in biomedical research (Cohen 2000; Carlsson *et al* 2004; Hau & Schapiro 2007; Fernstrom *et al* 2008). This necessitates their acquisition and importation from source countries in Asia and Africa, where animals are naturally occurring or where populations were introduced (Jones & Jennings 1994; Prescott 2001; Fernstrom *et al* 2008; Chapter 4).

Most purpose-bred cynomolgus macaques used for research purposes in GB are imported from overseas breeding centres in Mauritius, China, Indonesia, Philippines and Vietnam (Owen *at al* 1997; Prescott 2001; Prescott & Jennings 2004), with smaller numbers entering from Israel and Cambodia (Prescott 2001; Scientific Committee on Health and Environmental Risks 2009). Although the European Commission's Scientific Committee on Animal Health and Welfare (2002) raised concerns regarding the supply of primates to Europe from developing countries, the majority of cynomolgus macaques come into Great Britain from Mauritius (Prescott 2001; Weatherall 2006). The Home Office will only grant a project licence to use primates acquired from an accepted supplier[5] and they require pre-authorization of each animal consignment before transportation (Home Office 1989; APC 2006). Animals may be considered to be 'purpose bred' if they are F1 or F2 generation[6] (Scientific Committee on Health and Environmental Risks 2009), with

[4] At the time of submission there's a global excess of purpose-bred primates for biomedical research due to a decline in the pharmaceutical industry as a result of economic recession.
[5] The Home Office has a framework for acceptance including written information from the breeding establishment and site visits (APC 2006).
[6] F1 generation are the offspring of wild-caught (F0) animals, or F1xF0 parentage; F2 animals are the offspring of F1 x F1 longterm captive (FELASA 1999; Weatherall 2006; Scientific Committee on Health and Environmental Risks 2009).

increasing numbers of F2 animals being used (Weatherall 2006) as required under the new European Directive (EU 2010).

1.4 Trends in use of primates in Great Britain (GB)

1.4.1 Number of primates for research and testing in GB

The Home Office (HO) reports annually on the number of live animals used for research and testing under the Animals (Scientific Procedures) Act (1986). Figures 1.1(a-c) give HO statistics from 1999-2010 for the total number of primates used, further differentiated by major taxonomic divisions, and purpose of scientific procedures. Over the intervening eleven year period total primate use for research and testing has fluctuated annually (range: 2,649-3,354) and currently stands at 2,649; 0.07% of the total number of animals (Home Office 2011). Up until the onset of global recession (2008-09; International Monetary Fund 2009), the use of New World monkeys (NWM), principally marmosets and tamarins showed a steady decline from 1999-2008, whilst the number of OWMs, now exclusively macaques, collectively outnumbered their use by around 3:1 (Figure 1.1a). Over the same time-frame the numbers of OWMs were not declining but fluctuating around 2,500 per annum. Rennie and Buchanan-Smith (2005) noted a similar trend in the extent and character of primate use over an earlier period (1991-2002). Post 2008 however, a reversal of trends in the use of NWM and OWMs is observed, with the numbers of OWMs now under 2,000 for the first time in a decade. Cynomolgus macaques are most commonly used followed by rhesus macaques (*Macaca mulatta*; Weatherall 2006; Home Office 2011).

1.4.2 Procedures performed on primates

Figure 1.1b shows changes in the number of procedures using primates subdivided into Old and New World groups over the last decade. Some animals undergo more than one procedure if the previous procedure is classified as mild or moderate and the animal has recovered fully (re-use); hence the number of procedures exceeds the number of animals (Figure 1.1a). Following the decrease in their numbers, procedures using NWMs show a steady downward decline until 2008. Conversely it would appear more procedures were being performed per macaque, and despite annual fluctuations an upward trend was clearly visible until 2008 (Home Office 2011). This increase was likely to be due to the relatively low severity classification of toxicology procedures

(Sections 1.2). As with the number of primates being used, a reversal of trends in the number of procedures performed on NWM and OWMs is apparent. Primates are used for two main scientific purposes; toxicological (tox) or other safety and efficacy testing and nontoxicological (nontox), fundamental and applied studies (e.g. physiology, psychology, pathology, microbiology and pharmaceutical research and development; Figures 1.1c & d; Home Office 2000-2011). The greatest proportion (range: 80-93%; Figure 1.1d; Home Office 2000-2011) of procedures using macaques in the last eleven years were classified as toxicological, conducted to meet the regulatory requirements for approval of new pharmaceuticals in one or more countries (Home Office 2000-2011). This reflected a long-term trend in the increasing proportion of OWMs used in toxicity testing (Rennie & Buchanan-Smith 2005). These types of studies are collectively known as regulatory toxicology and most often carried out at Contract Research Organisations[7] (CROs) (APC 2002).

1.4.3 Macaque use in toxicology, safety and efficacy testing

Subchronic and subacute toxicological tests account for the largest percentage of toxicological procedures using macaques (Figure 1.1e). These tests involve repeated administration of the pharmaceutical under test (test article) for durations of at least three weeks (subacute) or at least three months (subchronic) (see Table 1.2) and form an integral part of preclinical safety testing (Parkinson *et al* 1996; Gad 2007; Walker *et al* 2007; Table 1.2). Although the overall severity of each toxicity test is not published in the annual HO statistics, all project licences for toxicology and general pharmaceutical safety assessment are prospectively banded for severity as mild[8] or moderate[9] (APC 2002). Special justification is sought from the Home Office for use of OWMs in toxicological procedures likely to exceed the mild severity banding (Home Office 1986). Moreover any project licence applications involving procedures likely to be of substantial[10] severity is referred to the Animal Procedures Committee[11] (APC) (Home Office 1986; APC 2009).

[7] The host and CASE sponsor for this PhD project is a CRO.
[8] Mild includes procedures that give rise to mild or transitory minor adverse effects.
[9] Moderate includes toxicity testing and surgical techniques that do not involve lethal endpoints.
[10] Substantial category is for procedures that result in a major departure from the animal's usual state of health and can involve major surgery, toxicity testing leading to death or the use of animals as disease models (Home Office 1986).
[11] The Animal Procedures Committee (APC) advises the Home Secretary on matters concerned with the Animals (Scientific Procedures) Act 1986. especially in relation to any experimental of scientific procedures applied to a protected animal that may have the effect of causing that animal pain, suffering, distress or lasting harm.

Figures 1.1a-e Trends in number of primates used, procedures performed and scientific purpose for research and testing in Great Britain (1999-2010).

Figure 1.1a Number of primates used for research and testing (1999–2010). Until 2008 the number of NWMs was decreasing over time; whilst the number of OWMs was fluctuating around 2,500 per annum. Post economic recession the numbers of NWMs are increasing whilst numbers of OWMs used for scientific purposes are decreasing.

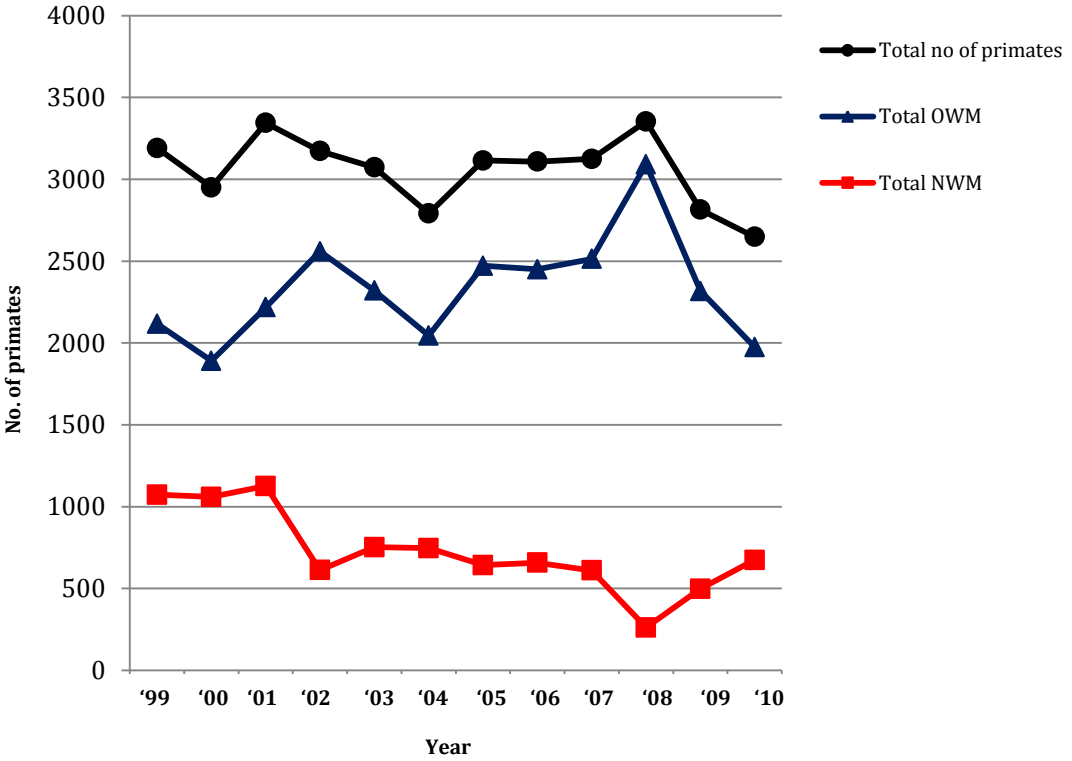


Figure 1.1b Number of scientific procedures using primates (1999–2010). The number of procedures on Old and New World monkeys was following different trends before 2008 and the onset of Global recession, with an increasing number of procedures being performed on OWMs each year. More recently the trends have reversed.

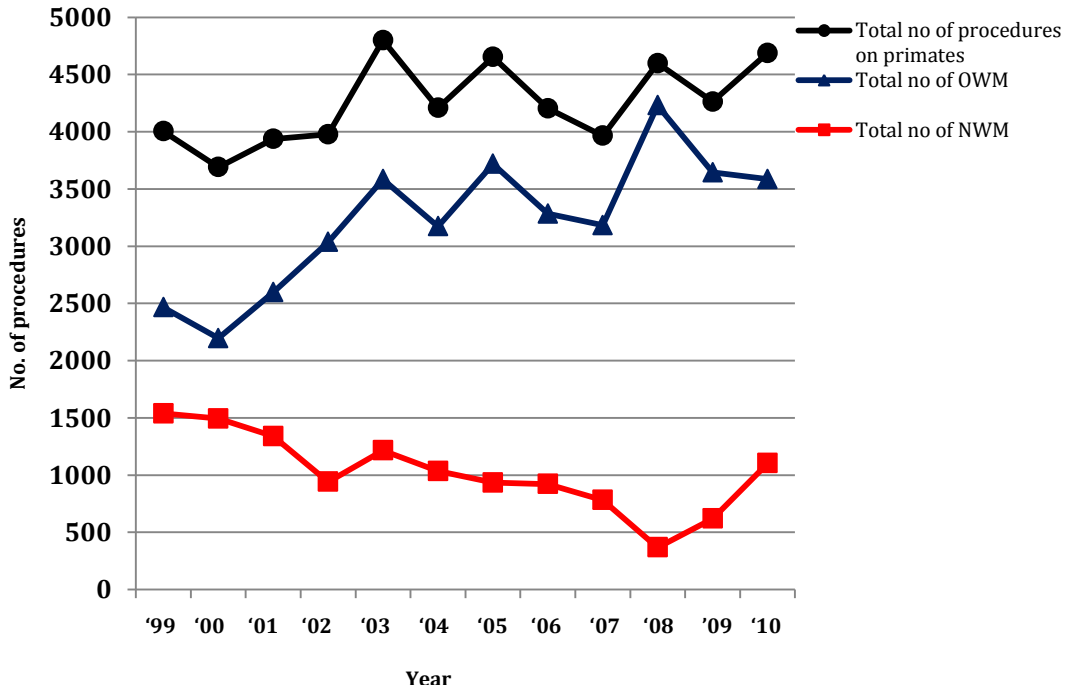


Figure 1.1c Number of primates used for toxicological (tox) and nontoxicological (nontox) scientific purposes. More primates are used for toxicological purposes than applied research (nontoxicological); OWMs are principally used in tox studies.

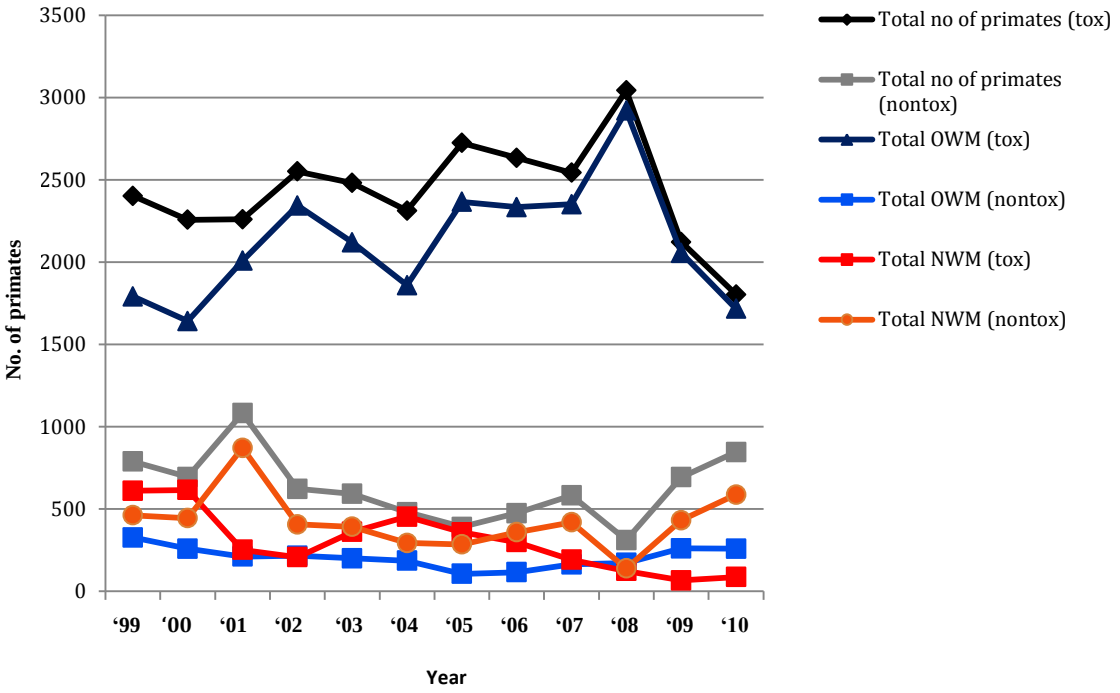


Figure 1.1d Percentage of toxicological (tox) and nontoxicological (nontox) procedures using macaques. The majority of procedures using OWMs were classified as toxicological.

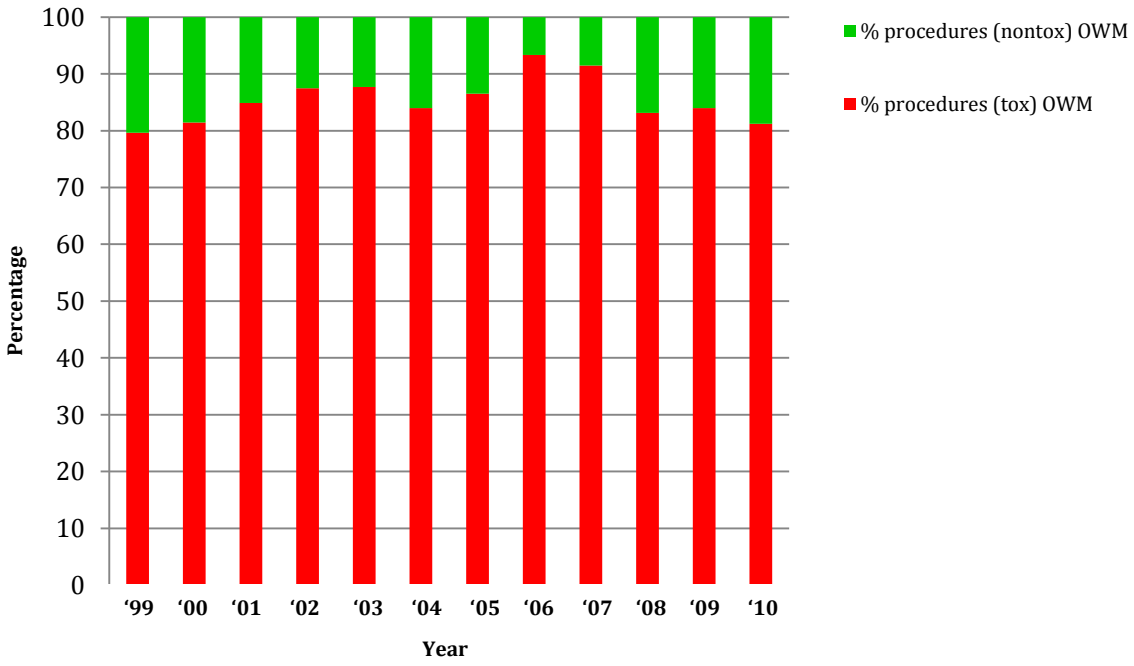
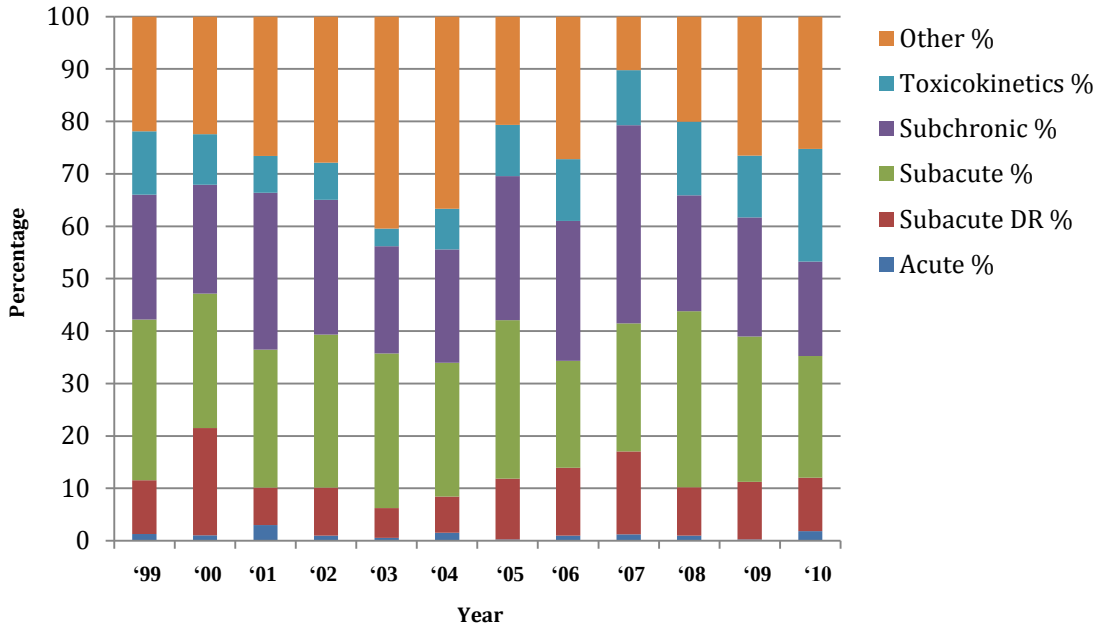


Figure 1.1e Percentage of procedures using macaques broken down into toxicological test. Subchronic and subacute toxicological tests account for the largest percentage of toxicological procedures using macaques.



1.5 Cynomolgus macaques as models for toxicology

Although not explicitly categorized in the statistics on animal use, cynomolgus macaques are the most frequently used macaque species in regulatory toxicology (Bleavins & de la Iglesia 2000; Scientific Committee on Animal Health & Welfare 2002; Scientific Committee on Health & Environmental Risks 2009; Keenan & Vidal 2006; Weatherall 2006; Walker *et al* 2007). The aim of regulatory toxicology in macaques is to characterise the effects of the test article on their biological systems (Helma 2005) at the molecular, cellular, tissue, organ, individual, and population levels, over a spectrum of effects; physiological, pathological and behavioural (Gad 2007) to enable risk assessment and determination of safe levels of exposure in humans (Zbinden 1991; Table 1.1).

Table 1.1 Three main tasks of experimental toxicology

Main tasks	Approach
Spectrum of toxicity	
- Detect adverse effects of chemicals in selected animal species. - Demonstrate dose-effect relationship over a broad range of doses.	- Assess alteration in physiological function, biochemistry and pathology, and relate to clinical signs *in vivo*. - Use a battery of measures. - Determine temporal relationships between recorded parameters, dosing and exposure.
Extrapolation	
- Predict adverse effects in other species, particularly humans.	- Evaluate via stepwise model scaling (see Gad 2007).
Safety	
- Predict safe levels of exposure in humans.	- Utilise integrated risk assessment: - Include biological data from preclinical and clinical trials.

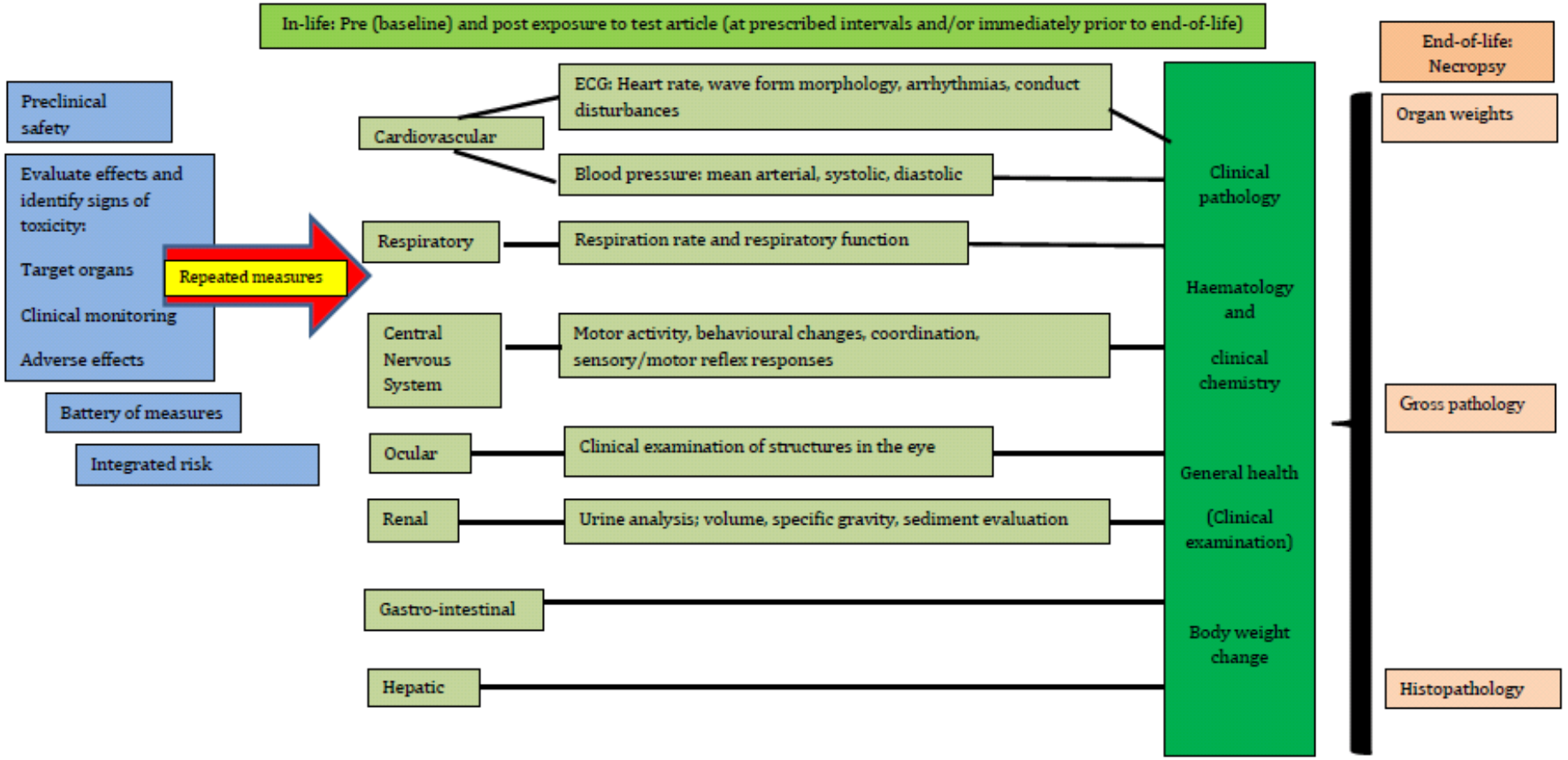
Adapted from Zbinden (1991).

1.5.1 Cynomolgus macaques as a *second* species

Guidance documents published by worldwide regulatory authorities (e.g. ICH: S7A, S7B, S6R1, S4, M3R1, ECB 2007 a & b) provide information on the development of appropriate study designs and recommend the inclusion of a core battery[12] of measures recorded both in-life and end-of-life (Section 1.5.3; Figure 1.2). They have a requirement to use a second, nonrodent species in testing prior to human exposure in clinical trials (ICH 1998; Council Directive 2001/83/EEC; APC 2002; EMEA 2006). Historically this has been the dog (Broadhead *et al* 1999) followed by the primate; the dog is considered the default second species and is actively ruled out before a primate can be considered (APC 2002; Home Office 2002; Smith & Trennery 2002). The extent to which this is achieved in practice has been questioned (Boyd Group 2002), and the cynomolgus macaque remains a popular choice for a number of other reasons, not least because of its similarity to humans with regard to their physiology, sensitivity to test article, metabolism and susceptibility to toxicity. Moreover macaque biology is well characterised through the availability of large amounts of background data (Walker *et al* 2007; Chapter 4).

[12] The term core battery is usually reserved for the battery of physiological measurements recorded in safety pharmacology, rather than those used in regulatory toxicology studies. The term has been used here and throughout the thesis for convenience.

Figure 1.2 Schematic view of preclinical safety evaluation using macaques as an example for toxicity testing to meet regulatory requirements.



1.5.2 Practical considerations

In addition to macaques' physiological and anatomical closeness to humans, the practicalities of test article administration by a number of desired routes (Gad 2007), ease of collection of body fluids, blood samples and recording of physiological parameters (a requirement in the core battery) in relation to body size are also taken into consideration (Hall & Everds 2008). Ethical concerns, limited supply, cost of procurement and housing further drives the need to maximise the information collected from each animal (Hall & Everds 2003). For example their relatively large blood volumes (estimated 80ml/kg; Kamis & Noor 1981) permit serial blood sampling for a variety of blood analyses (Chapter 4). The resulting toxicology study with multiple dosing and sampling procedures, and associated handling and restraint is a likely welfare concern (Chapters 3, 4 & 5). Although most toxicology studies are relatively well controlled (Hall & Everds 2008) many procedures and variables impact on interpretation of data collected from the few animals tested (Hall & Everds 2003; Chapters 2 & 4).

Table 1.2 Overview of toxicological, safety and efficacy testing in the drug development process. Recommendations in the conduct of preclinical trials to support human clinical trials.

	Study type	Description
Preclinical	Safety pharmacology	Safety pharmacology includes the assessment of effects on vital functions: Cardiovascular, central nervous and respiratory systems. The effects should be evaluated prior to human exposure. Can be conducted as additions to toxicity studies or as separate studies.
	Toxicokinetic & pharmacokinetic	Exposure data in animals should be evaluated prior to human clinical trials. Additional information on absorption, distribution, metabolism and excretion (ADME) in animals should be included and available by the completion of Phase I (Human Pharmacology) studies.
	Single dose toxicity	Single dose (acute) toxicity for a test article should be evaluated in **two mammalian species** prior to the first human exposure.
	Repeated dose toxicity studies	Recommended duration of repeated dose toxicity studies is related to the duration, therapeutic indication and scale of the proposed clinical trial. In principle, the duration of the animal toxicity studies conducted in **two mammalian species** (rodent and nonrodent) should be equal to or exceed the duration of the human clinical trials up to the maximum recommended duration of the repeated dose toxicity studies.
	Local tolerance	Local tolerance should be studied in animals using routes relevant to the proposed clinical administration. The assessment of local tolerance may be part of other toxicity studies.
	Reproduction toxicity studies	Reproduction toxicity studies should be conducted as is appropriate for the population that is to be exposed.
	Study type	**Description**
Repeated dose toxicity in **two species (rodent and nonrodent**) for a minimum duration of 2 weeks would support Phase I (Human Pharmacology) and Phase II (Therapeutic Exploratory) studies up to 2 weeks in duration. 1, 3 or 6 months toxicity studies support these types of human clinical trials for up to 1, 3 or 6 months, respectively. Six month rodent and chronic **nonroden**t studies would support clinical trials of longer duration than 6 months.		
Clinical	Phase I	*Human pharmacology studies* Conducted on healthy volunteers.
	Phase II	*Therapeutic exploratory studies* Conducted in patients and healthy volunteers, exploratory efficacy and safety studies.
For the Phase III (Therapeutic Confirmatory) studies in Europe: One month toxicity study in **two species (rodent and nonrodent)** would support clinical trials of up to 2 weeks duration. Three month toxicity studies would support clinical trials for up to 1 month duration. Six month toxicity studies in rodents and 3 month studies in **nonrodents** would support clinical trials of duration up to 3 months. Longer term clinical trials, a 6 month carcinogenic study in rodents and a chronic study in **nonrodents** are recommended.		

Clinical	Phase III	*Therapeutic confirmatory studies* Clinical patients, confirmatory clinical trials for efficacy and safety testing in the patient population.

Adapted from ICH Topic M3 (EMEA 2000). The length of chronic studies in rodents and nonrodents will depend upon the proposed clinical testing and therapeutic regimen of the drug in the end (human) patient.

1.5.3 A core battery of measures

A core battery of measures (e.g. electrocardiograph, blood pressure, standardized lists of hematology and clinical chemistry and urinalysis parameters) are included to assess potential target organ toxicity (James 1993). Adverse clinical (physical) signs including fluctuations in animal body weight provide information on the clinically observable symptomology and therefore potential signs that may require monitoring during testing in humans (Walker *et al* 2007).

The cost and practical limitations of surgical implantation for telemetered and chronic dosing devices necessitate administration of a test article (dosing) and recording the core battery in regulatory toxicology studies whilst animals are manually restrained rather than being conducted remotely as is often the case with safety pharmacology (e.g. ICH Topics S 7 A). Walker *et al* (2007) gives common dosing and sampling routes for primates used in preclinical toxicology, with their associated handling and restraint techniques. Capture, handling and restraint are known to be stressful for primates (JWGR 2009; Chapters 3, 4 & 5). The handling-associated arousal that results can not only adversely impact on welfare but is also likely to affect the physiology of the research subject and therefore the validity of research data collected (Chapters 2, 3, 4 & 5). There are however, many opportunities to Refine housing, husbandry, handling, restraint and procedures for macaques in toxicology (e.g. Home Office 1986; Diehl *et al* 2001; Morton *et al* 2001, Boyd Group 2002; JWGR 2009). The host establishment for this PhD project recognises this and devotes considerable resources and time to improving the lives of their animals and quality of scientific measures (e.g. Kelly 2007; 2008; 2009 Chapters 4 & 5).

1.5.4 Study design

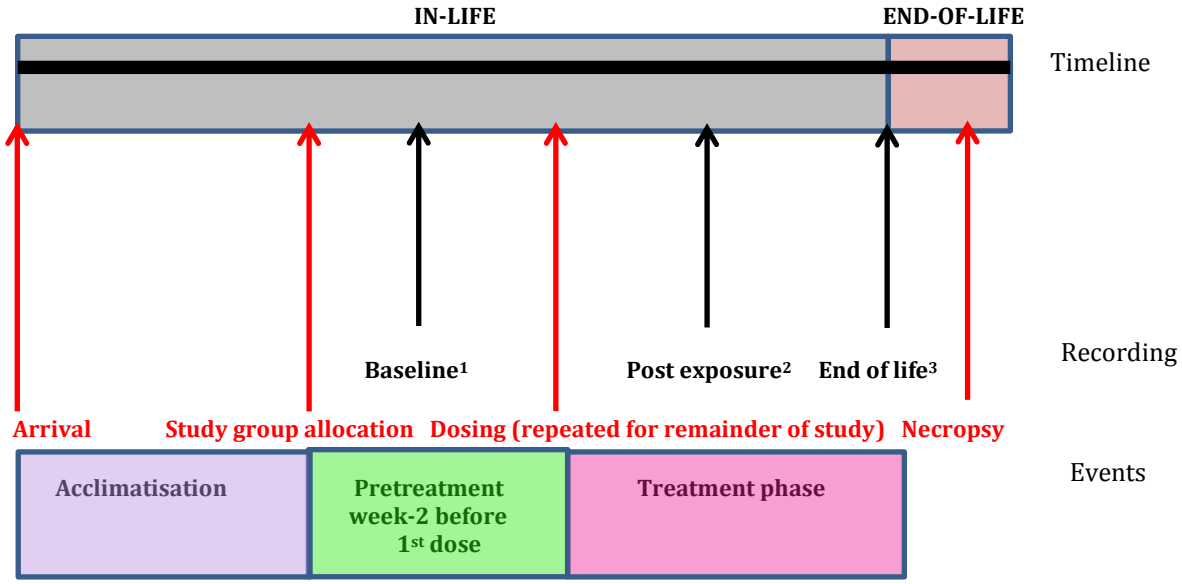
Ultimately the final design of a preclinical toxicology study depends on the intended therapeutic use of the test article in humans that is dose level, dose volume, dose route, frequency of dosing,

duration of study and frequency and timing of the core battery (Walker *et al* 2007). Primate toxicology studies must be conducted in-line with A(SP)A (1986) and those studies adhering to Good Laboratory Practice (GLP)[13] require approved protocols, detailing specific collection and analysis methods, and defined lists of in-life and end-of-life evaluations.

Typically four groups of animals are required; a concurrent control group, subject to the same procedures and sham dosed (often with a vehicle not containing the test article), and three dose groups; given low, mid and high dose levels (Walker *et al* 2007). The lowest dose is typically greater than the expected human dose; it should demonstrate the No Observed Effect Level (NOEL), that is no observable signs of toxicity. The highest dose is some multiple of the proposed clinical human dose and should induce toxicity without causing morbidity or mortality (Robinson *et al* 2009). The middose is an intermediate level usually with minimal toxicity to provide characterization of a dose response to the test article (Walker *et al* 2007; Robinson *et al* 2009). The number of animals in each group varies from 2-6 (Gad 2007). A repeated measures design (Figure 1.3), with animals acting as their own control allows for quantification of the toxicological effects on the animals' physiology. Statistical analyses are performed on group mean values for each parameter measured in the core battery and for each recording occasion (James 1993). The final results from the core battery are related to each other to identify target organ toxicity and to understand underlying mechanisms and the time course of toxic changes (James 1993).

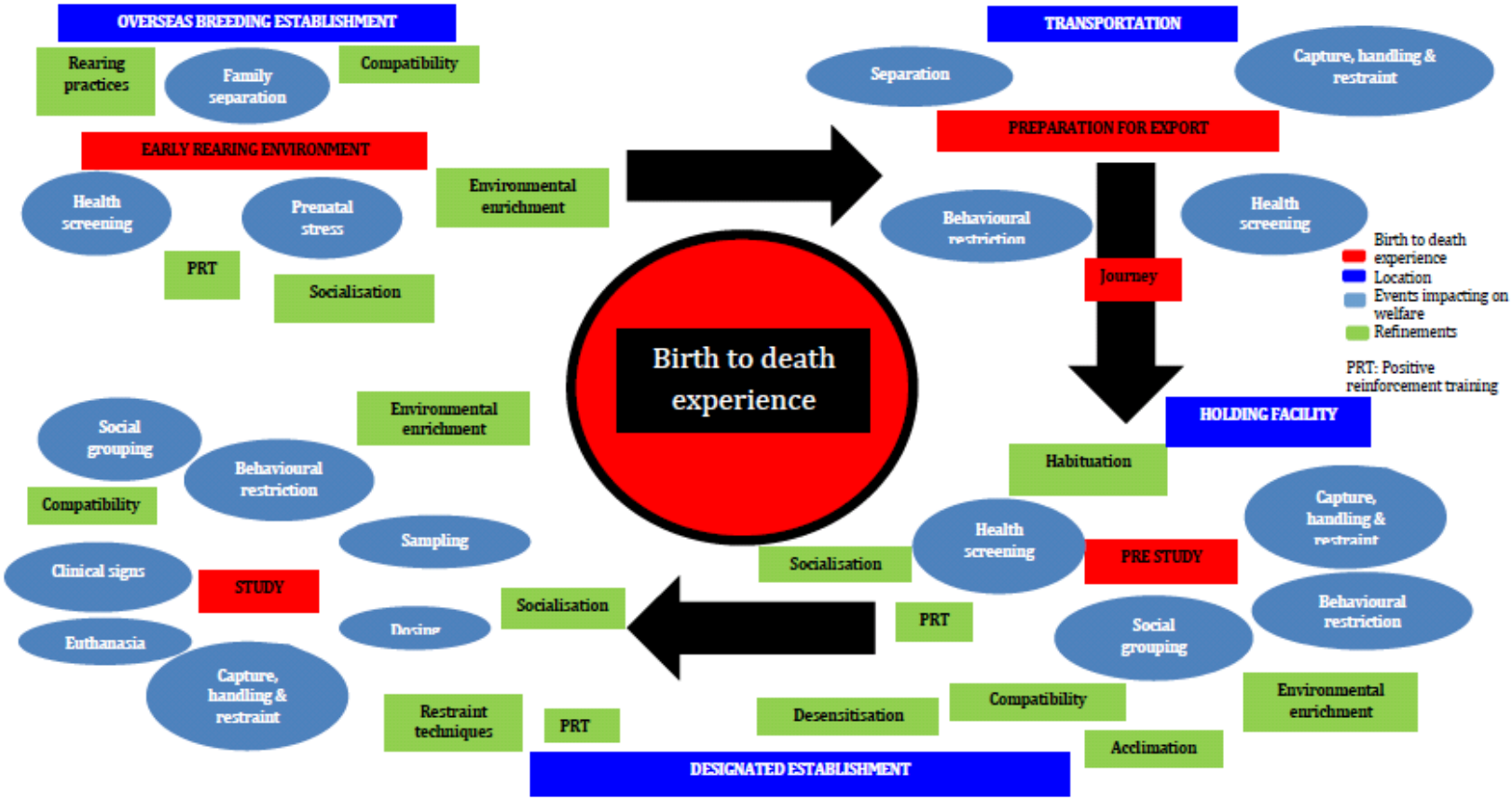
[13] Good Laboratory Practice (GLP): Legal requirement in Organisation for Economic Co-operation and Development (OECD) countries. Laboratories carrying out studies submitted to national authorities are required to follow GLP guidelines for the purpose of assessment of chemicals in relation to protection of man and the environment. Guidelines promote the quality and validity of test data used for determining the safety of chemicals and chemical products.

Figure 1.3 Schematic presentation of events in a regulatory toxicology study. Not to scale.



[1]Baseline procedures: First recording of the core battery (ECG, BP, H&CC; Figure 1.2); body weight recorded at least weekly from arrival and termed 'pretreatment' from week -2 before first dose: [2]Post exposure procedures: multiple recordings of core battery at prescribed intervals. Time and frequency of repeated measures are dictated by the test article and intended clinical therapeutic use: [3]Immediately prior to end-of-life all or part of core battery are recorded.

Figure 1.4 Key events that typically occur in the life cycle of cynomolgus macaques used for regulatory toxicology with potential for Refinement. It does not include re-use.



1.6 Ethical considerations

1.6.1 Ethical issues surrounding the use of primates for research and testing

The use of primates for research and testing is contentious (see Prescott 2010 for review) particularly amongst the general public as illustrated by a recent European Commission's public consultation on animal experiments, where more than 80% of respondents considered the use of primates to be not acceptable (EC 2006). This public concern is reflected in the text of the new European Directive 2010/63/EU (e.g. EU 2010, p L276/34-35; Olsson & Vitale 2010); the use of primates and the availability of alternatives will be subject to periodic review (EU 2010, p L276/51).

The fundamental ethical dilemma surrounding the use of all animals for experimental purposes is whether humans are morally justified in causing pain, suffering, distress or lasting harm during research aimed at alleviating or preventing human suffering (Rollin 2007). All vertebrate animals may suffer during exposure to painful, distressing procedures (Olsson *et al* 2003), and their housing in the laboratory is often behaviourally restrictive, whilst handling, restraint and care-staff contact may cause physical discomfort and psychological distress - the extent of which depends upon the species in question (Olsson *et al* 2003). Additionally, primates' phylogenetic closeness to humans, the scientific justification for their use as research models (Weatherall 2006; Scientific Committee on Health and Environmental Risks 2009), is argued to make them a special case (see Boyd Group 2002), and is used by opponents as a reason to prohibit their use in laboratories (Balls 2000; Eurogroup for Animal Welfare 2005).

Central to this argument is that primates may suffer in a different way than other animals and in more similar ways to humans (Boyd Group 2002; Nuffield Council on Bioethics 2005; Weatherall 2006). Whilst they share anatomical and physiological features with humans, they also have complex cognitive, psychological and social needs (see Boyd group 2002 for review), which have implications for the nature of their suffering in the laboratory (Boyd Group 2002; Nuffield Council on Bioethics 2005; Weatherall 2006; Buchanan-Smith 2010; Prescott 2010). Indeed this is recognised somewhat at a legal level in that primates along with dogs, cats and equidae are afforded special protection (Home Office 1986). Yet it is methodologically difficult for scientists to

compare the suffering of primates in laboratories to that of other commonly-used species (Boyd Group 2002; Webster *et al* 2010). The core difficulty lies in understanding the subjective experiences of primates in their capacity to suffer. However, their evolutionary closeness to humans and physiological, neurological and behavioural similarities does permit us to make important approximations (Prescott 2010). Furthermore we can demonstrate that their welfare is affected by housing, husbandry and procedures in the laboratory (JWGR 2009; Chapters 3, 4 & 5), which has important implications for their scientific use (Chapters 2, 4 & 5). Whether primates should be protected above all other animals (See Webster *et al* 2010 for discussion) or given the same moral status as humans in relation to their use in scientific use is subject of on-going debate (Quigley 2007).

1.6.2 Ethical frameworks, legal provisions and regulatory guidelines

The Utilitarian approach is adopted for dealing with the ethical dilemma of using animals in research and testing, and one which is enshrined in British and European legislation, and underpins many local ethical review processes (Home Office 1986; EEC 1986; EU 2010). This pragmatic approach weighs the ethical importance of the individual and their capacity to suffer against the interests of the other parties concerned (Singer 1975; Sandøe *et al* 1997). In practice this approach is known as a harm-benefit assessment; it is currently applied to primate use prospectively at the project licence level (Home Office 1986). The perceived 'harm' to the animal in terms of its likely experience of pain, distress or lasting harm including likely intensity, duration and frequency, is weighed against the anticipated benefits of the research for humans (or other animals or the environment) (Section 1.1). Similarly this approach will be used in retrospective reporting of projects where the actual costs to the animal will be reviewed in-light of the results of scientific study (EU 2010; Section 1.1).

Primates are used in efficacy and safety testing of new pharmaceuticals provided that it is (a) scientifically demonstrated that (b) no alternative rodent and/or nonrodent species is appropriate for the purpose of the study (Smith & Trennery 2002; ICH 2009). They are most often used as a second species to meet the regulatory requirements after the dog; however the increasing development of monoclonal antibodies as therapies for life threatening human disease means that

they may be the only relevant animal model in preclinical safety testing owing to the high target- and species- specificity that these small molecules demonstrate (see Chapman *et al* 2009; 2010 for review). Therefore the numbers of primates being used worldwide is increasing with in-line with rising numbers of biologics entering the drug development pipeline (Chapman *et al* 2009), although they are not always relevant for predicting human safety (Chapman *et al* 2009).

In addition to requiring assessment of the harms and benefits of proposed research, the legislative framework also requires the 3Rs principle be applied to the project from its experimental design to its execution (Home Office 1986; EEC 1986; EU 2010). Not only do these propositions for humane science have considerable scientific merit (e.g. Refinement; Chapters 2, 4 & 5) but they can also help increase public support for animal research (Ipsos Mori 2009). Despite the widespread scientific support of the 3Rs principle this does not always translate into effective implementation (NC3Rs 2008). Prescott (2010) reviews the opportunities for applying the 3Rs principle to research using primates. More specifically the application of Refinements in housing, husbandry and procedures for primates used in regulatory toxicology are discussed in Chapters 4 & 5.

Where their use is indicated, scientific, animal welfare and practical considerations should be taken into account with harm-benefit analysis and application of the 3Rs so that primates are only used when absolutely necessary, when morally justified, and animal suffering is kept to a minimum (Prescott 2010). In Chapter 2 I explore the link between welfare, Refinement and scientific output which further emphasizes that because of primates' capacity to suffer, it is critical that their welfare is maximised and that their use results in the most reliable, repeatable and valid experimental outcomes.

2

Linking welfare and quality of scientific output in toxicology

"*It has sometimes seemed that there is an irreconcilable conflict between the claims of science and medicine and those of humanity in our treatment of lower animals……The conflict disappears altogether on closer inspection, and by now it is widely recognized that the humanest possible treatment of experimental animals, far from being an obstacle, is actually a prerequisite for successful animal experiment*"

Russell & Burch (1959), p 3 - 4.

Zbinden[1] (1985; p 138) writing on "*The special responsibility of the toxicologist*":
"*As scientists they must…… question whether the methods and concepts that currently form the basis of experimental toxicology are still valid. Having done this they must consider carefully the routine experimental procedure and ask themselves, whether they really get the best, the most comprehensive and the most relevant information from each animal they kill in the name of human safety. If they are not convinced that their current approaches are indeed the best to protect human health against chemical injury, they have an obligation to make their doubts known, to initiate well directed research programmes and to press for revision of regulatory requirements should their investigations demonstrate that new methodological approaches provide more relevant results*."

[1]Zbinden is credited with translating "*the 3R's (reduction, refinement, replacement) of Russell and Burch into the language of the toxicologist*" Alan M. Goldberg, Director of the Center for Alternatives to Animal Testing at John Hopkins University, (Witschi 2000).

Abstract

*The link between good welfare and good science is often made but with little qualification about what we mean by 'good', 'welfare', and 'science' and with few supporting quantitative data. The concept of animal welfare is considered in terms of three dimensions; how the animal feels, based on it leading a natural life, and its biological functioning. In the laboratory, animal welfare centres on Refinement which includes both the reduction of negative welfare states (e.g. pain, suffering, distress) and the proactive enhancement of positive welfare over the animal's lifetime. There's a growing body of evidence linking poor welfare and unsound experimental outcomes, but studies aimed at proactively enhancing welfare and systematically examining the link with experimental results in toxicology, are sparse, particularly for commonly used cynomolgus macaques (*Macaca fascicularis*). Toxicology is concerned with the characterisation of effects of new drugs on the biological systems of animal models prior to human exposure. The concept of quality of science in this case should focus on scientific output. The assumptions are that toxicology provides an answer to an important problem, cynomolgus macaques are a relevant model, the core battery of biological variables are valid measures of physiological function and pathology, and that toxicological investigations in macaques can predict risk of adverse effects in humans. Meeting these* priori *conditions, the question is: with enhanced welfare do we improve quality of scientific outcomes? This is approached in two ways. First, do we observe measurable improvements in sensitivity of individual biological variables, that is do we record baseline measures closer to normal and potentially improve our ability to determine magnitude and direction of changes in physiological responses with chronic dosing? Second, do we enhance the reliability and repeatability of toxicological measures, by reducing the variation within and between animals? These dimensions of quality of scientific output are used to examine the link between welfare and science in regulatory toxicology for the thesis.*

2.1 Introduction

It is of ethical (Section 1.6.2) and scientific (Section 2.3) importance that macaque welfare is safeguarded in the laboratory. The complex cognitive, psychological, social and physical needs of macaques mean they experience negative welfare states, including stress as a result of the restrictive nature of the laboratory environment and in response to potential harms during study. Stress has wide-ranging effects on biological variables, often the same ones of interests to toxicologists (e.g. heart rate, blood pressure, haematological and biochemical parameters etc.), and may lead to reduced welfare (Section 2.2.4). The behavioural and physiological changes during stress responses (Chapters 3, 4 & 5) may confound our ability to quantify the adverse effects of chronic drug treatment as part of regulatory studies (Section 1.5.3).

Maintaining high welfare depends upon having an understanding of what welfare is (Section 2.2), how it can be assessed (Chapter 3), and a strategy for rapid implementation of changes and their evaluation. Underlying this process should be an acceptance that this is a necessary, on-going, and circular process - it is a permanent challenge for care staff and scientists to advance best practice. Indeed the study of animal welfare is founded on the assumption that humans have an ethical obligation to the animals whose lives they influence and they must continually strive to improve their welfare (Sandøe *et al* 1997; Appleby 1999). Discussions of animal welfare in the literature are based on three approaches; feelings-based, ability to lead a natural life, and biological functioning (Section 2.2). These concepts have shaped the respective methodologies for investigation, they often overlap, and a more holistic approach seeks to integrate them in assessments of animal welfare (Section 2.2.5; Chapter 3). In the laboratory, welfare is formally considered in terms of Refinement, one of the three Rs synonymous with humane science enshrined in legislation and codes of practice (Section 1.2). The concept of Refinement has evolved beyond just a reduction of harms to animals, as proposed initially by Russell and Burch (1959) and now seeks to proactively enhance welfare over the animal's life time (Buchanan-Smith *et al* 2005).

The link between good welfare and good science is commonly quoted throughout the literature, regulatory guidelines, and legislation, and whilst the evidence for poor welfare and poor experimental outcomes is well known (Section 2.3; 2.4), a reduction in negative welfare state does

not necessarily result in good welfare; welfare may be less bad, but not good. The link between good welfare and good science remains somewhat unexplored, especially in cynomolgus macaques (*Macaca fascicularis*), an important nonrodent model used for safety and efficacy testing of new drugs (Section 1.4.3). Further, the concept of quality of science has many dimensions, and is the subject of a resurgence in toxicology, especially with regard to the use of historic data sets for modelling drug effects (Chapter 4) and in the validation of alternative methods, to reduce animal numbers in testing (Section 2.4). In order to evaluate quality of science for this PhD study, piggy-backing on toxicology studies at a contract research organisation, I adopted a much narrower concept focusing on scientific output and accompanying fundamental aspects (Table 2.2) such as sensitivity, reliability, and repeatability of individual measures in the core battery (Sections 2.4.2 & 2.4.3).

In this Chapter I discuss the concepts of animal welfare, quality of scientific output and the link between the two. I propose a framework for examining the link in the thesis (Section 2.5) with special reference to cynomolgus macaques in regulatory toxicology.

2.2 Animal welfare

Animal welfare has been the focus of scientific study for over thirty years (Fraser *et al* 1997); yet constructing a single definition and approach to measurement has been difficult (Duncan 1981; Mason & Mendl 1993; Duncan & Fraser 1997; reviewed in Fraser 2009). It is accepted that welfare is broad in concept, multidimensional in nature (Dawkins 2004), and lies on a continuum from poor to good (Broom 1999). Three approaches, based upon the relative importance of factors attributed to the concept of animal welfare (Duncan & Fraser 1997) are summarised below. They include how the animal *feels* (Duncan & Petherick 1991; Duncan 1996; Simonsen 1996; Dawkins 1998; Section 2.2.1), whether the animal is able to lead a *natural life* (Duncan & Petherick 1991; Duncan 1996; Simonsen 1996; Section 2.2.2) and an approach based upon the animals' *biological functioning* (Broom 1986; Curtis 1997; Fraser & Broom 1990; Broom 1996; Section 2.2.3; Chapter 3).

In the laboratory the concept of animal welfare is formalised in Refinement (Section 1.2), initially concerned with decreasing the adverse effects of experimental procedures on animals, defined as "*simply to reduce to an absolute minimum the amount of stress imposed on those animals that are still used*" (Russell & Burch 1959, p 134), it has evolved to include both the reduction of negative welfare states (e.g. pain, suffering, distress) and proactive enhancement of positive welfare over the animal's lifetime and includes experiences out with procedures (see Buchanan-Smith *et al* 2005; Rennie & Buchanan-Smith 2006a,b; JWGR 2009; Figure 1.4). Similarly for farm animals, changes in attitudes mean the concept of welfare includes the promotion of positive welfare (e.g. Yeates & Main 2008; Napolitano *et al* 2009; Westerath *et al* 2009). Species-specific indicators of positive and negative welfare for cynomolgus macaques are discussed in Chapter 3.

2.2.1 Feelings-based

Concerns over animal welfare are often founded on the assumption that non-human animals can experience negative and positive emotional states and hence suffer or experience pleasure (Dawkins 1990; Duncan & Fraser 1997; Mendl 2001; Mendl & Paul 2004; Paul *et al* 2005). Indeed for experimental animals it is the potential for scientific procedures to cause pain, suffering or distress that causes greatest concern (Aldhouse *et al* 1999; Plous 1999) and negative emotional states form critical components in the definition of animal welfare (Duncan & Petherick 1991; Mason 1991; Duncan 1996; Simonsen 1996; Duncan & Fraser 1997; Dawkins 1998; Fraser & Duncan 1998). The relatively new focus on positive welfare means that it has less clearly refined terminology and physiological correlates (Boissy *et al* 2007; Yeates & Main 2008; Table 2.1).

For some it is intrinsically difficult to investigate directly, the subjective, emotional experiences of animals (Mason & Mendl 1993; Barnard & Hurst 1996; Duncan 1996; Mendl *et al* 2009) whilst others passionately advocate otherwise (see below; Wemelsfelder 1997).

Table 2.1 Definitions of positive and negative subjective states

	Subjective state	Definition	Author
Affect		"Involves positive and negative feelings."	Yeates & Main 2008, p294.
Negative	**Pain**	"An aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal's physiology and behaviour to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery; unnecessary pain occurs when the intensity or duration of the experience is not appropriate for the damage sustained or when the physiological and behavioural responses to it are unsuccessful at alleviating it."	Molony & Kent 1997, p266.
	Painful procedure	"Any procedure that would reasonably be expected to cause more than slight or momentary pain or distress in a human being to which the procedure was applied, that is, pain in excess of that caused by injections or other minor procedures."	Institutional Animal Care & Use Committee 1997, AC 11.1.
	Suffering	"A negative emotional state which derives from adverse physical, physiological and psychological circumstances in accordance with the cognitive capacity of the species and of an individual being and its life experience."	Morton & Hau 2002, p458.
	Stress	"Biological response elicited when an individual perceives a threat to its homeostasis. The threat is the 'stressor'." "When the stress response truly threatens the animal's well-being, then the animal experiences distress."	Moberg 2000, p1.
	Distress	"An aversive, negative state in which coping and adaptation processes fail to return an organism to physiological and/or psychological homeostasis."	Moberg 2000, p3.
	Fear	"An emotional reaction induced by perception of stimuli associated with danger which leads to protective defence reactions."	Janczak 2010, p251.
	Anxiety	""Fear responses are shown primarily to well-defined threats; whereas anxiety is shown in healthy animals when the source of the threat is less clearly defined."	Janczak 2010, p32.
	Boredom	"Resulting from chronic lack of opportunity of active interaction with the environment."	Wemelsfelder 2005, p79.
	Frustration	"Results when an animal is prevented from performing behaviour that it is motivated to perform."	Keeling & Jenson 2009, p98.
Positive	**Liking**	"The affective consequence of a reward: the positive feelings experienced by an animal."	Yeates & Main 2008, p294
	Wanting	"A mental state associated with a motivation to gain a resource or effect an interaction."	Yeates & Main 2008, p294.
	Happy	Defined operationally (specifically for macaques see Chapter 3). "Alert and busy (displays a wide behavioural repertoire), is able to rest in a relaxed manner, is confident (outward going and does not display fear towards non-threatening stimuli) and does not display abnormal behaviour."	Poole 1997, p116.
	Pleasure	"Any sort of positive experience whose basis could be physical, emotional, or both"	Balcombe 2011, p 3.

Historically, and with less emphasis in the literature latterly, preference and operant testing (reviewed in Kirkden & Pajor 2006) have given some insights into how animals feel about: given housing conditions (Dawkins 1980; 1991), performance of particular behaviours (Duncan & Hughes 1988; Fraser & Matthews 1997), accessing specific resources (Matthews & Ladewig 1994) and avoiding certain situations (Rushen 1986). Animals are deemed as preferring an option if they spend more time within it, or they choose it more often or they show a shorter latency to approach it (Bateson 2004). These methods are limited to relative and often short-term motivational priorities or preferences and don't necessarily indicate the strength of the animal's motivation or reflect long-term consequences of their performance (Duncan & Fraser 1997; Bateson 2004). Consumer demand theory provided an alternative approach in an attempt to ascertain how

strongly an animal feels about a given choice. Testing often involved using operant apparatus to determine the "price" an animal will pay (e.g. the weight of a push door, the number of lever pushes performed) to escape, avoid or achieve access to a given situation; the higher the 'price' willing to be paid, the stronger the preference (Dawkins 1990).

There are a number of limitations with both these approaches, especially when considering whether providing animals with preferred options will lead to actual improvements in animal welfare. The application of results from testing paradigms to animals *in-situ* is far from straightforward (reviewed by Bateson 2004; Kirkden & Pajor 2006). Firstly animal preferences may be dependent upon the precise context in which a preference is measured (Bateson 2004). Indeed, internal (e.g. previous experience; Dawkins 1977, Grandin *et al* 1994; reproductive state; Cooper & Appleby 1995) and external (e.g. temperature; Fraser 1985, time of day; Fraser & Matthews 1997) variables, and social isolation (Pedersen *et al* 2002; Sherwin 2003) have been found to affect animals' expressed preferences. Yet animals live in a continually changing environment and as a result neither the internal milieu nor external stimuli are static. Not only that, testing paradigms merely establishes preference along a single motivational dimension (Nicol 1997) and thus may not be relevant to complex, and multifactorial situations outside the context in which the choice experiment was performed (Mason *et al* 1997). These restrictions make it difficult to ascertain whether providing animals with the preferred option leads to a reduction of suffering (Dawkins 1983).

More recently animal welfare scientists have explored novel approaches measuring animals' emotional states (reviewed by Olsson *et al* 2011); quantitatively, by evaluating changes in cognitive information processing ('cognitive bias' e.g. Paul *et al* 2005; Mendl *et al* 2009) or qualitatively, by assessing observers' interpretation of animals' (attributes of) emotional expression ('Qualitative Behavioural Assessment: QBA' e.g. Wemelsfelder *et al* 2001; 2009).

The term 'cognitive' broadly refers to information processing in humans and non-human animals such as attention, learning, memory and decision-making. It can influence and be influenced by the individual's emotional state (reviewed by Mendl *et al* 2009). When responding to an event or

stimulus, an appraisal, a series of 'mental' checks are undertaken (e.g. is the stimulus novel or familiar, pleasant or unpleasant, sudden or predictable?), and an evaluation (reference to memories), such that the valence (whether emotional states are positive or negative for the animal) can bias the ensuing cognitive response, which in turn can be measured (reviewed by Paul *et al* 2005; Mendl *et al* 2009). For example, animals in a more negative emotional state are more likely to be 'pessimistic' (Matheson *et al* 2008) when presented with an ambiguous stimulus compared to animals in a more positive state that display more 'optimistic' judgements (Harding *et al* 2004). Whilst behavioural and physiological responses (Section 2.2.3) are good measures of emotional arousal (e.g. intensity) they are not necessarily good measures of emotional valence (e.g. direction - positive or negative) as fear and pleasure evoking stimuli may result in the same physiological or behavioural response regardless of the animal's underlying emotion (Mendl & Paul 2004; Paul *et al* 2005; Mendl *et al* 2009). The cognitive bias approach has been found to produce consistent findings across animal species (reviewed in Mendl *et al* 2009).

For example a recent study examined the effects of husbandry events on the emotional state of rhesus monkeys (*Macaca mulatta*) (Bethell *et al* 2012). Two common husbandry procedures; provision of additional enrichment and chemical restraint for veterinary inspection, were considered salient stimuli, such that they exert either negative (e.g. restraint) or positive (e.g. enrichment) effects on monkeys. The testing paradigm involved training seven adult male monkeys to associate responding (e.g. touching a computer screen) to a presented visual stimulus (length of yellow line on a computer screen) with either a reward (long line) or no reward (short line). Subsequent testing, in a repeated-measures design, compared number of touch screen responses post exposure to the two husbandry procedures. By recording the animal's response to presentation of three ambiguous stimuli (e.g. varying lengths of yellow line; one being most similar to the long line - associated with reward, the second was similar to the short line - no reward, and the third, intermediate in size, was most ambiguous), the researchers got an indication of whether monkeys were more 'optimistic' (touched the longer line) or 'pessimistic' (touched the shorter line) following restraint or enrichment. Bethell *et al* 2012 found differential shifts in emotion state following the two husbandry procedures, such that they influenced the monkey's judgements about the positive or negative meaning of the presented ambiguous stimuli (yellow lines of varying

lengths). Monkeys were found to be more likely to touch the longer yellow lines than the shorter ones (e.g. lines closer in length to those associated with a reward during initial training) following enrichment when compared to the post restraint condition, demonstrating a negative shift in cognitive bias following restraint relative to additional enrichment. This finding indicates a cognitive change occurred with emotional valence post stressor (Bethell *et al* 2007; 2012).

A somewhat different, qualitative, approach pioneered by Wemelsfelder and co-workers (e.g. Wemelsfelder *et al* 2001; 2009; Wemelsfelder & Farish 2004; Rousing & Wemelsfelder 2006), also aims to advance our understanding about how animals feel. Known as Qualitative Behavioural Assessment (QBA), observers are asked to qualitatively assess animal's body language. Using a numerical scale or their own descriptors (e.g. content or anxious) observers characterise the animal's emotional expression (Wemelsfelder *et al* 2001; Wemelsfelder & Farish 2004). This qualitative approach has shown good agreement with quantitative measures of welfare (e.g. behaviour, Rousing & Wemelsfelder 2006; and physiological measures - heart rate and heart rate variability, Wemelsfelder 2007). Yet despite the advances and promise of such feelings-based approaches, they are criticised as being too narrow in focus. Indeed an animal may feel happy, but if it has a terminal disease, which will kill it, even though it may be in a pre-pathological state we would not say it had good welfare (Section 2.2.3). Nonetheless subjective feelings and the performance of natural behaviour (Section 2.2.2) are adaptive and often promote biological functioning (Duncan & Fraser 1997; Section 2.2.5). Indeed animals which are able to perform behaviours that they are strongly motivated to carry out will likely feel better (Novak & Drewsen 1989; Duncan & Fraser 1997), and it is this approach I shall discuss next.

2.2.2 Leading a natural life

The natural-living approach (e.g. Fox 1983; Rollin 1993) considers welfare in terms of whether animals can express their 'natures' or '*teleos*' (Rollin 1993), species typical behaviours they have evolved to perform (Duncan & Fraser 1997; Morton 2003) in their natural environment (Fox 1983). Further, animals have '*behavioural needs*', a term used to describe a fundamental requirement in the biology of the animal to carry out activities (Poole 1992). It is termed 'need' for good reason - it is not an option or luxury (Ewbank 1985) for the animal to be able to perform it,

but a necessity, by which we mean that the animal is strongly motivated, internally driven, to perform it in order to obtain a particular resource, or respond to a bodily stimulus or environmental cue, and if prevented from doing so its welfare is jeopardized (Hughes & Duncan 1988; Broom & Johnson 1993; Duncan 1998). Even domesticated species (e.g. farm and companion animals) have retained a repertoire of natural behaviour that their ancestors living in the wild evolved through a process of natural selection (Duncan 1998). In spite of this retained behaviour serving little or no function in the captive environment its performance is probably very important to the welfare of the animal (Duncan 1998). Keeping animals in captivity therefore, in environments, which share few physical features compared to their natural environments, may result in frustration and suffering. To promote welfare, we should raise and keep animals in natural environments where they can display their full behavioural repertoire (wild counterparts living under natural conditions are used as a benchmark for comparison; Kiley-Worthington 1989; Novak & Drewsen 1989; Lindberg & Coe 1995; Duncan & Fraser 1997).

Indeed the observation of natural behaviour in captive conditions is usually desirable (Shepherdson 1990; Chapters 3 & 4). It suggests that the environment shares similar characteristics to natural habitats, for which animals have evolved anatomical, behavioural and physiological adaptations (Chapters 3 & 4), and can be used as an indicator of whether the environment meets the animal's behavioural needs (Poole 1992).

There are however, a number of drawbacks with this approach. Firstly, the laboratory environment can never fully mimic dynamic conditions in the wild; it is incompatible with standardization, and the widely fluctuating environmental conditions would introduce unwanted variation into experimental results (Table 2.2; Section 2.4.1). Secondly, the operational definition of this approach is not clear. For example how should we characterise the range in species typical behaviour in terms of poor or good welfare. Should it be described in terms of its occurrence, frequency and duration or quantity by which it deviates from wild counterparts? (Novak & Drewsen 1989). Which wild populations should we use for comparison given that individual, genetic, geographic and temporal variations occur (see Veasey *et al* 1996a & b for discussion)?

Moreover, natural environments do not always offer the best quality of life for wild animals. A full behavioural repertoire would include behaviours related to adverse events (Dawkins 1980), such as escape from predation, hunger, illness, infanticide and injurious aggression etc. Animals experiencing these could not be described as having good welfare (Novak & Drewsen 1989; Poole 1996). Further, the performance of new behaviours or behaviours in different contexts by laboratory-housed populations may indicate behavioural flexibility and an adaptation to the different environment, this is not necessarily a sign of reduced welfare (Veasey *et al* 1996b). Therefore the assumption that natural behaviour is an indicator of good welfare can sometimes be impractical and the significance of its performance for animal welfare is not always clear (Rosenblum 1991; Veasey *et al* 1996a & b; Dawkins 1998). Nevertheless, the study of wild populations in natural habitats can give us an important benchmark for the assessment of the welfare of laboratory animals through understanding their communication mechanisms (Chapter 3) and their biological and environmental requirements (e.g. in relation to housing and care; Röder & Timmermans 2002), for gaining ideas to improve the laboratory environment (e.g. environmental enrichment; Mellen *et al* 1998; Roush *et al* 1992; JWGR 2009; Buchanan-Smith 2010), and for appreciating the normal breadth of the range and frequency of behaviours animals have evolved to perform (Poole 1992; 1996).

2.2.3 Biological functioning

Animals live in a continually changing environment, in order to survive environmental challenges, physiological and behavioural feedback mechanisms maintain an internal equilibrium, and physiological variables are kept at their 'set point', a process known as homeostasis (Frandson & Spurgeon 1992; Korte *et al* 2007). This approach to welfare is concerned with the animal's *biological functioning* (Broom 1986; 1996; Curtis 1987; Fraser & Broom 1990) and thus "*the welfare of an animal is its state as regards its attempts to cope with its environment*" (Broom 1986). When an animal is unable to cope with prolonged or intense environmental challenges its normal biological function may be disrupted (Moberg 1985), resulting in disease, body damage, behavioural pathology and reduced production measures (Broom 1991; Broom & Johnson 1993; Pedersen 1996); as a consequence the animals' welfare is diminished (Fraser & Broom 1990). Disease, injury and abnormal behaviour are easier for observers to quantify than other welfare

parameters (Duncan & Fraser 1997) and often these are the types of parameters recorded on a regular basis by care staff in the laboratory (Chapter 3).

Korte *et al* (2007) suggest that the concept of welfare, in relation to homeostasis, proposed by Broom (1986) is now out-dated because it ignores the absence of environmental challenges which produce under (hypo) stimulation; the consequences of which may be poor welfare. Instead the concept of allostasis is proposed as it involves mechanisms that change the physiological variable by predicting what level will be needed to meet anticipated demand (Sterling & Eyer 1988). Thus an unusual (high or low) physiological value is not considered as a failure to protect a narrow set point (as in homeostasis) but rather as a response to some prediction (Sterling 2004). The emotional brain plays a central role in allostasis (Koob & Le Moal 2001), such that previous experience, feelings, memories etc., are coordinated in anticipation of physiological requirements (Korte *et al* 2007). A shift in demand results in a shift in the coordinated response (Sterling 2004), the release of hormones, neurotransmitters and cytokines working at tissue and organ level to produce adaptive changes to metabolism, immune and cardiovascular systems in the short term (McEwen 1998; Korte 2001; McEwan & Lasley 2002; Korte *el al* 2005).

Natural selection has shaped physiological and behavioural responses to meet the most likely environmental demands (predictions) with a small safety margin (see Korte *et al* 2007 for review). A fit animal has a wide regulatory range of allostatic mechanisms (Korte *et al* 2007) and activation of these mechanisms outside of this range can result in failure to habituate with repeated challenges such that a shift in physiological response is seen so that the animal continues to respond once the challenge is over, or an adequate response is not mounted (Korte 2001). This results in a state of chronic deviation of the regulatory system from its normal operating system and this new allostatic state is characterised by narrower regulatory ranges with the potential for over or under stimulation (Korte *et al* 2007). When an allostatic load is chronically high pathologies may develop (McEwen & Lasley 2002) and under stimulation results in a very low allostatic load characterised by different pathologies such as depression, fatigue, allergic reactions and autoimmune disorders (Sternberg 1997).

Good animal welfare is therefore characterised by a broad predictive physiological and behavioural capacity to anticipate environmental challenge (Korte *et al* 2007). In captive animals however, housing conditions usually require extensive adaptation, because they do not meet the environmental demands for which the animal has adapted (Chapter 4) resulting in an allostatic state characterised by reduced regulatory capacity (Korte *et al* 2007). Often the irreversible nature of changes in reactivity and resilience and the resultant damage to tissues and organs can be used a measure of allostatic load and hence welfare (Korte 2001; Koob & Le Moal 2001; Koob 2004; Korte *et al* 2005).

2.2.4 The link between stress and welfare

Stress and welfare are closely associated concepts (Wiepkema & Koolhaas 1992; 1993; Veissier & Boissy 2007). They share similar characteristics: both can be described in terms of physiological and behavioural responses (e.g. heart rate, blood pressure, cortisol release, flight, avoidance) which often overlap; the intensity of response is considered to reflect degrees of averseness (measured along a continuum), and they have a cognitive component whereby the response is dependent upon the animals' perception and appraisal of the situation (Wiepkema & Koolhaas 1992; 1993; Veisser & Boissy 2007). They're considered in opposite terms such that good welfare cannot be achieved under stress (Veissier & Boissy 2007). The concept of stress was once considered to be non-specific, known as the General Adaption Syndrome (Selye 1973). It is now known that different stressors evoke specific stress responses (behavioural and physiological) (Mason 1971; Levine *et al* 1989) but physiological responses often show ceiling and floor effects (Section 2.4.2) where by their range (upper and lower) are limited by biological constraints (e.g. heart rate, blood pressure and cortisol; Chapter 5), which makes it difficult to reliably distinguish between higher levels of responses to higher intensity of stress or welfare experienced and vice versa lowered responses (Veissier & Boissy 2007).

Animals have adapted to survive in a variable world (Chapter 4), novelty and uncertainty in the natural environment evoke a stress response but their quality and quantity are within the range of the animals' coping capabilities. What's more, some environmental instability/uncertainty is necessary to avoid boredom (Wemelsfelder 1990) and optimize individual vigilance. The difficulty

lies in distinguishing the limits of what's acceptable for an individual's welfare. Under conditions exceeding the animal's capacity to cope, symptoms of stress may arise that precede or reflect a pathological state and welfare is therefore threatened (Moberg 1985; 2000).

Nearly all stressors have one characteristic in common, the psychological impact of control and predictability (Wiepkema & Koolhaas 1992; 1993). The interactions between the two are complex and outside the realm of discussion for the thesis (see Bassett & Buchanan-Smith 2007 for discussion). However as a general rule, the greatest behavioural and physiological influence would be seen in animals that have neither control nor predictability over an event. Animals that could either predict or control the event lie somewhere in between the former and latter; animals that can predict and control the event show the least severe response (Overmier *et al* 1980). Wiepkema and Koolhaas (1993) argue that for optimal welfare some uncertainty that is some unpredictability and/or uncontrollability is positive; complete uncertainty may lead to monotony resulting in boredom (Wemelsfelder 1990).

2.2.5 Integrated approaches for animal welfare

Rather than considering each of these approaches independently, our understanding of animal welfare may benefit from how the respective methodologies interrelate (Nicol 2009) and in combination (through multiple measures) they provide a logical framework for the interpretation and validation of indicators of welfare (Broom 1986; Curtis 1987; Novak & Suomi 1988; Fraser & Broom 1990; Broom 1996; Crockett 1998; Dawkins 1998; Fraser & Duncan 1998; Chapter 3). Thus, for an integrated approach to the concept and assessment of welfare Dawkins (2003; 2004) has suggested we should be concerned with two aspects - is the animal healthy and does it have want it wants? Similarly, Webster (2005, p252) has also come up with a succinct way of capturing physical and mental aspects of animal welfare, asking whether the animal is "*fit and feeling good*". The rationale and use of specific welfare indicators for cynomolgus macaques used in regulatory toxicology is given in Chapter 3.

2.3 Scientific importance of welfare

Russell and Burch (1959) were the first to describe the scientific basis for humane treatment of animals involved in biomedical experimentation. Here too the focus lay squarely on the link with inhumanity and experimental outcomes, and less focus was placed on promotion of good welfare. In part this was limited by a lack of understanding of positive welfare states at the time. Nonetheless the scientific importance of welfare has continued to gather momentum (e.g. Poole 1997), such that fifty years later it is near impossible to read text books on care of laboratory animals, toxicological methods, legislation, regulatory guidelines and codes of practice without a mention of good welfare and good science. In order to examine the link between the two, we need to discuss what we mean by quality of science. Before we proceed, we assume that experiments using animals are answering important scientific questions; in terms of toxicology, that a non-animal alternative was not possible and cynomolgus macaques are a relevant predictive model to identify risk of adverse effects in humans. Further assumptions include the core battery of biological variables to be valid measures of physiological function and pathology, and that the experiments are well-designed (reduction techniques applied), efficiently executed, correctly analysed and interpreted (Festing & Altman 2002). With specific reference to toxicology the discussion of quality appears below.

2.4 Quality of scientific output

Given the concern and uncertainty about the moral acceptability of using nonhuman primates in medical experiments because of the evolutionary proximity to human beings (Section 1.6.1), the case for the use of non-human primates in research is receiving much attention (Weatherall 2006). Recent reviews and assessment of outcomes of studies using primates centre on quality of experimental design and impact on research (see Prescott *et al* 2010; Bateson *et al* 2011). The term quality in its broadest sense describes conformance to requirements (Crosby 1979), fitness for use (Juran 1974) or "*the standard of something as measured against other things of a similar kind; the degree of excellence of something*" (Oxford Dictionary of English 2003). Similarly, quality in science too is concerned with fitness for use: an experiment yielding unambiguous results by minimising unwanted variation and absence of confounding factors; providing answers to an important problem (Poole 1997); conformance to requirements; and undertaken to a required

standard (e.g. Good Laboratory Practice and International Conference on Harmonisation guidelines). In addition to aspects of quality pertaining to the study, quality in its fullest sense, should also include the impact of research using animals (see Bateson *et al* 2011) – that is the application of knowledge resulting from research findings.

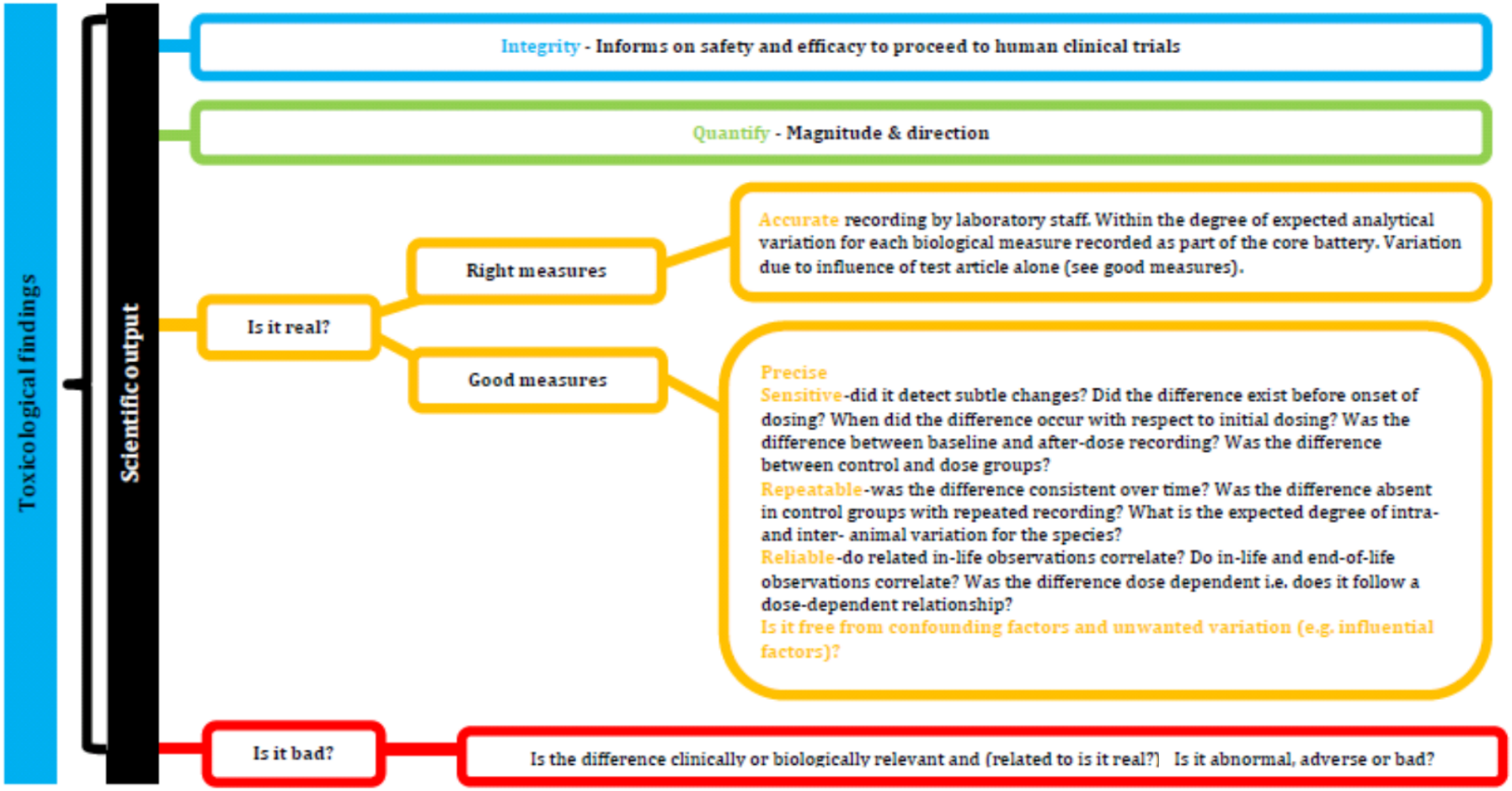
Assessment of scientific quality is often by peers (e.g. Bateson *et al* 2011); acceptance of research results in peer-reviewed journals (Johnson & Besselsen 2002), expertly evaluating specified methods, robustness and importance of findings, providing the review panel have the full breadth of knowledge and experience (e.g. Bateson *et al* 2011 and see Kilkenny *et al* 2009; Prescott *et al* 2010). When measured on a global scale using various bibliometric indices (e.g. number of published articles, citation indices; see Groneberg-Kloft *et al* 2009; Loscalzo 2011; Bateson *et al* 2011) it provides a relative (but imprecise) way to evaluate impact of research activities. This approach is not appropriate for assessing the quality of regulatory toxicology studies, because aside from the inherent difficulties regarding lack of information and transparency of reporting in the scientific literature (see Kilkenny *et al* 2009; Prescott *et al* 2010; Bateson *et al* 2011) the results of such studies are rarely published; they are reported to the sponsor and regulatory body. This makes it difficult to fully assess the impact of such studies (see above).

A narrower concept of quality, focusing on scientific output (Table 2.2) was adopted for this discussion in relation to toxicology, which is concerned with characterising the effects of a new drug candidate (test article) on the biological systems of an animal model (e.g. cynomolgus macaque) to meet regulatory requirements (Section 1.4.2). As described in Chapter 1, serial measurements of many dependent variables (core battery) are made on individual animals assigned to dose groups. Any or all of the core battery may be altered by differing quantities and directions by the test article under study (Festing & Altman 2002). The biological response, that is data generated during testing, must be converted into information before they become meaningful for risk assessments prior to human exposure (Purchase *et al* 1989). In addition to quantifying the size and direction of the change, Hall (1997) poses two important questions: first, is the difference real? That is, can the response be attributed to the test article and that alone? Second, is it bad? Is the finding abnormal, adverse or bad for the given test species? Furthermore, the biological

response in animals must be converted into more meaningful information to assess safety and efficacy prior to human exposure (Purchase *et al* 1989). For example, animal data are entered into a quantitative model or algorithm which enables toxicologists to extrapolate preclinical findings (e.g. NOEL; Chapter 1), deriving a safe human equivalent dose before onset of *first-in-human* clinical trials (US FDA 2005).

When considering these two questions it is pertinent to consider scientific quality (Figure 2.1), determined by the appropriateness of the study design to answer the questions of interest and the care with which the study was performed (Rodricks & Turnbull 2007). Good Laboratory Practice (1991) is the mandatory quality standard for preclinical toxicology; it is a quality system concerned with the organisation process and conditions under which studies are planned, performed, monitored, recorded, archived and reported (OECD 1997). It assures study integrity (Table 2.2), sound study management and traceability (Seiler 2001). It does not, however, influence scientific aspects of study planning and conduct (Spindler & Seiler 2002). These in part are assured by adhering to guidelines for study design outlined by the International Conference on Harmonization (ICH) which include the following recommendations: use groups of healthy animals, acclimatised to laboratory conditions, housed under specified and standardized conditions, allocated to study groups by randomization procedures, with a concurrent control group (sham dosed with vehicle), and record test batteries designed to measure biological effects, recorded under standardized methods specified in standard operating procedures. Both GLP and ICH guidelines assume that scientifically valid methods are used, and that measures to protect reliability of preclinical toxicity studies are undertaken (Spindler & Seiler 2002), but the definition of quality and aspects pertaining to it in the context of preclinical toxicity studies are not explicitly defined.

Figure 2.1 The relationship between toxicological findings in animals (see Hall 1997) and quality of scientific output (Table 2.2 gives definitions of terms). Animal welfare impacts on scientific output (e.g. is it real?; Section 2.4.2-3).
Assumptions: cynomolgus macaque is a relevant animal model and that a core battery of tests are valid measures of biological functioning.



Assessment of quality of toxicological information is receiving renewed attention (e.g. Hartung 2009) with a drive to reduce the number of animals used in *in vivo* testing. For example, under REACH, the use of existing toxicological information is emphasised (Hoffman *et al* 2008) for predictive toxicology (Gottman *et al* 2001; Cronin 2005) and in the validation of alternative test methods (ICCVAM 1997). Previous attempts to rate the reliability of existing toxicological data (e.g. Klimisch *et al* 1997) have somewhat ill-defined criteria, and tools for assessing reliability of data are being developed (Hoffman *et al* 2008; Schneider *et al* 2009). Nevertheless, the assumption remains that conforming to prescribed standards (e.g. GLP) and accepted study approaches (e.g. ICH), ensures quality in scientific output (Gottman *et al* 2001).

Table 2.2 provides an overview of dimensions of quality of scientific output. The aim was to clarify definitions which would enable a framework for examining the link between Refinement, welfare and quality of scientific output (Section 2.5; Figure 2.1) – the core theme of this thesis. In some cases the dimensions are closely related and even overlap. The two central concepts, sensitivity and variation (in relation to reliability and repeatability), are then explored in greater detail, and in relation to animal welfare.

Table 2.2 Definition and explanation of terms used to describe quality of scientific outcomes.

	Term	Definition		Explanation	Related & contributing factors	Protection, assumptions and methods of promotion
		Oxford Dictionary	Scientific			
Global terms	**Integrity**	The quality of being honest; lack of corruption.	**The degree to which data collected and reported are what they purport to be (14).**	**A combination of study management and scientific method quality.**	Validity Accuracy Precision Reliability Repeatability	**Protected to a degree by compliance with GLP, following guidelines on study conduct and reporting by regulatory bodies (e.g. 17).**
	Relevance	Closely connected to; Appropriate to the matter in hand.	The extent to which a test method correctly predicts or measures the biological effect of interest (12); appropriateness of tests and/or data for a particular hazard identification or risk characterisation (16).	The scientific basis to support the method (6); Establishing the scientific meaningfulness and usefulness of results for a particular purpose (1-4; 11 & 12).	Validity Accuracy Precision Reliability Repeatability	Prediction of toxicity is a complex process, dependent on: the selected animal model, the experimental design, data collection methods and the methods of extrapolation (13).
Right measures	Validity	*Valid* – actually supporting the intended point or claim.	The extent to which a measurement actually measures what the scientist wishes to measure and provides information to the questions being asked (16); Reliability and relevance of the method in supporting a specific use (2;3 & 17).	A valid or 'right' measure refers to the relationship between the variable under study and what it is supposed to measure or predict about the world. Valid measures are those that actually answer the questions being asked. Implies the experiment has a high probability of meeting the stated objectives (9); The objectives have a reasonable chance of contributing to human or animal welfare (9). Whether data derived can be generalised to other species- external validity (16)	Accuracy Specificity Relevance	Assumptions: (a) cynomolgus macaques are a relevant model; (b) the dose selection and route of administration match the intended target species (c) frequency and timing of data collection are based upon prior knowledge of drug action (d) core battery of biological variables are valid measures of organ function, histology, and pathology etc. Data must accurately represent the toxic endpoint being assessed (7).
	Accuracy	The degree to which the result of measurement, calculations, or specification conforms to the correct value or a standard.	Closeness to the real value (8); The degree to which measured/ calculated values reflect the true values of what they intend to represent (14); The closeness of agreement between a test result and an accepted reference value (12).	Is the measurement unbiased (free from systematic errors[a]), such that measured values correspond with true values? (16).	Validity Precision Specificity	Protected by compliance with quality assurance schemes (Section 2.4): instrumentation and analytical methods are accurate within, specified limits. Note reference values for comparison are influenced by environmental and animal factors (Chapter 4).
	Specificity	Identify clearly and definitely; Precise and clearly repeatable.	To what extent does the measure describe what it is supposed to describe and nothing else i.e. the ability to detect true measures (16).	Is the measurement describing single or multiple: biological variable(s) and function(s); specific for pathology and free from interference from external variables?	Robustness Unwanted variation Confounding factors	Assume limitations of current analytical methods are known and specified.

Good measures	**Reliability** **Section 2.4.3**	Consistently good in quality or performance.	**The extent to which the measurement is repeatable and consistent (free from systematic errors[a]). Unbiased measurement, represents the true value of the variable, reduces the random component from imperfections in the measurement process (16). A reliable method produces results that are accurate and correctly reflect the sample being tested (15).**	**The smaller the error component the more reliable the measurement (16).**	Precision Sensitivity Accuracy Precision	**If the measurement is unreliable the real effects (Figure 2.1) of the test article are difficult to quantify. This has implications for interpreting whether findings are biologically relevant i.e. are they bad? (Figure 2.1) and risk posed prior to human exposure.**
	Precision	The quality, condition or fact of being exact and accurate.	How free the measurements are from random errors[b] (16); Closeness of repeated measure to same value (8).	A measure of the reproducibility of the predictions of a model or repeated measurements (14).	Reproducibility Variation Accuracy	Reported for regulatory bodies.
	Sensitivity **Section 2.4.2**	Quick to detect or respond to slight changes, signals or influences.	**The ability to reliably measure small changes, clearly distinguishable from background noise (14).**	**Are small changes in the true value reflected by changes in the measured value?(16)**	Accuracy Precision Reliability	**Depends on the sensitivity of the model to the toxicity of the test article – model and test article dependent. Lower and upper limits of determination are specified for analytical methods. Determining small changes in biological function may be impaired if the variation (background noise) is great (Chapter 4) or the physiological parameter is constrained by a ceiling or floor effects (e.g. Chapters 4 & 5).**
	Repeatability **Section 2.4.3**	Occur again in the same way and form.	**The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions with a given method (12); Can be described for measurements conducted repeatedly on a single individual or animals (within/intra-) or between individuals sampled at the same time in the same way (between/inter-).**	**The test result should be repeatable any number of times with low error (7).**	Robustness Reproducibility Influential factors	**Requirement for physiological parameters to be relatively stable over the given study time frame to enable a real difference (Figure 2.1) in response to test article to be detected. Related to standardization, acclimatisation (Section 2.5).**
	Reproducibility	Produce a copy of, with a specified degree of success.	The degree to which a given method is reproducible within and between laboratories (12).	The biological endpoint must be reproducible. The same result is obtained if the test is repeated in other laboratories (7).	Repeatability Robustness	Most often used when developing alternative methods.

	Robustness	Sturdy in construction; Strong, vigorous.	The insensibility of a test method to departures from the specified test conditions when conducted in different laboratories (12).	Robust method is one where successful results are obtained a high percentage of the time (13).	Specificity Reproducibility Repeatability	Most often used when developing alternative methods.
Influential factors	**Variation Section 2.4.3**	Change or slight difference in condition, amount, or level, typically within certain limits.	**Term used to describe heterogenecity of values over time, space or different members of a given populations. An inherent property of the system or population (14).Intra-individual variability (difference over time in the same individual); inter-individual variability (differences between members of the population); Controllable variable –deliberately controlled for in the population, forms part of a control strategy in the method (14).**	**Any set of observations or measurements derived from a group of individuals will exhibit variability (1).**	Accuracy Specificity Precision Repeatability Unwanted variation Confounding factor	**Biological variation follows a normal distribution; source, handling, restraint and environmental conditions can affect variation in the population (e.g. confounding factor; Chapter 4) – effects meaningful comparison to historic/background data. Wanted variation describes the variation in response to test article. See below:**
	Undesirable variation	*-undesirable variation* (variation as above)	Undesirable or unintended variation (16).	Skilful experimenter will attempt to eliminate these sources of variation (16).	Standardization	Use of a concurrent control group and comparable baseline data to identify undesirable variation.
	Confounding factor Section 2.4.1	*Confound-* Cause confusion; Mix up (something) with something else.	**A confounding effect is caused by any other factor outside of the experimental treatment that might be present in the experimental arena (8).**	**Environmental or husbandry factors are examples of confounding factors that will impair ability to interpret study data (Section 2.5; Chapters 4 & 5).**	Standardization	**Use of controls, randomization, and standard operating procedures to minimise the risk of confounding factors giving false negative or positive results. See standardization.**
	Standardization Section 2.4.2a	To conform to a standard; A required or agreed level of quality or attainment.	**Setting standards; Defines properties of experimental animals and their environments to increase reproducibility of results (5); Keeping experimental conditions the same for all animals (within-experimental standardisation) or all experiments (between-experimental standardisation) (19).**	**Measures are taken to reduce variation relating to source, sex, environmental conditions, data capture and analysis by following standard operating procedures.**		**Trade off with external validity i.e. our ability to extrapolate results to other populations (Section 2.5). Previously used as an argument against group housing, environmental enrichment, socialisation and training programmes for laboratory housed animals (Chapters 4 & 5).**

[a]Systematic error: avoidable error due to controllable variables in a measurement; [b]Random error: Unavoidable errors that are always present in any measurement. Impossible to eliminate.
References (in alphabetical order): 1: Balls *et al* 1990a; 2: Balls *et al* 1990b; 3: Balls *et al* 1990c; 4: Balls *et al* 1995a; 5: Beynen *et al* 2001; 6: Bruner *et al* 1996; 7: Cronin 2005; 8: Dytham 2003; 9: Festing & Altman 2002; 10: Frazer 1990; 11: Gauch 2003; 12: ICCVAM 1997; 13: IPCS 1978; 14: IPCS 2008; 15: Klimisch *et al* 1997; 16: Martin & Bateson 2007; 17: OECD 1996; 18: Russell & Burch 1959; 19: Wurbel 2002.

2.4.1 Good laboratory science is based on normal, healthy, happy animals

Poole's (1997), seminal paper '*Happy animals make good science',* argues that good laboratory animal science is based upon normal, healthy and happy animals, unless illness or alleviation of stress is the subject of study. When considering this in terms of toxicology, the most obvious starting point of study would be an animal that's healthy, in a stable state and normal (discussed below). These concepts are obviously advantageous for a repeated measures design that aims to identify adverse effects of a test article on an animal's biological system by quantifying effects of repeated dosing when compared to baseline measures. However, they are not necessarily as straightforward as they first seem (see discussion below) and perhaps the most controversial, happiness, requires further explanation, yet it is integral to enhanced psychological well-being, and we now recognise how interdependent psychological and physiological systems are. The concept of 'happiness' therefore, cannot be ignored when considering good quality laboratory science including toxicology.

Indeed Poole (1997) appears to be writing way ahead of his time, discussing this positive affective state (Section 2.2.1; Table 2.1), whilst others focussed on Refinement as a way of minimising suffering, rather than the proactive enhancement of positive welfare, now central to its evolved definition (Buchanan-Smith *et al* 2005). He defines a happy animal as "*one which is alert and busy (shows a wide repertoire of behaviour), is able to rest in a relaxed manner, is confident (outward going and does not display fear towards trivial non-threatening stimuli) and does not show abnormal behaviour,*"p116. This is an operational definition and gives an obvious framework with which to use behavioural measures to determine whether an animal is 'happy' (Chapter 3). Happiness is not an absolute binary concept, as with other concepts and measurements integral to animal welfare (subjective feelings being one of them), it can be discussed in relative terms and on a continuum from good to bad - unhappy to happy, getting happier etc. Happiness therefore is inherent for good welfare as it encompasses positive affective states (Table 2.1). In Chapter 3 we revisit the operational definition given by Poole (1997), outlining correlates of physical health and physiological parameters that link with species-specific behavioural measures.

Scientific method assumes the absence of confounding factors and uncontrolled variables (Poole 1997). Whilst the effects of disease are easy to identify, and their confounding effects are well known (Festing 2002; Hall 2007; Rodricks & Turnbull 2007), Poole (1997) argues that unhappiness is also a confounding variable because its effects on biological variables produces increased variation. He goes further asserting that most scientists working with animals assume that they have normal physiology and behaviour (e.g. heart rates, blood pressure, blood values, metabolism, hormones, immunological competence etc.). However, the physiological, biochemical and behavioural parameters of an animal can be dramatically altered by the conditions in which they were bred, reared, kept, transported and the way in which experimental procedures were conducted (Chapters 3, 4 & 5), which experimenters assume to be normal because they commonly encounter them and have no reference for comparison. For example, historic control databases which are used to construct reference intervals of biological parameters in cynomolgus macaques contain data recorded from animals handled, restrained and housed in conditions that are not conducive to good welfare. These data are considered 'normal' because they statistically represent 95% of the population from which they have been derived (Chapter 4). Yet animals with poorer welfare are considered normal and their data a benchmark for comparison. It is only when we begin to examine changes in the laboratory environment that we begin to recognise that normal limits change with enhanced welfare (Chapters 4 & 5). Our concept of normal should also be defined and considered context-specific and indeed derived normal ranges may be nothing like what's normal or optimal for the natural animal. With lower basal physiological values, we are less likely to experience the confounding influence of ceiling effects (reviewed in Chapter 5) and improve the sensitivity (Table 2.2) of biological variables in response to dosing, to enable accurate determination of the magnitude and direction of any changes.

In addition to requiring normal physiology and behaviour for good science, toxicological studies assume that baseline measures are stable and that time spent in the laboratory is not a confounding factor with serial measurements. To avoid this bias it is recommended as a minimum that animals are acclimatised and habituated to procedures (Section 2.2.3b) before onset of study (e.g. ICH). The problem with this approach is that it is often laboratory-specific (Chapter 4 outlines recommended time scales) and prescriptive (based on inputs e.g. number of prior experiences,

length of time in the laboratory) but rarely undertaken with a view to meeting predetermined outputs (e.g. demonstration that biological parameters have normalized and stabilized; Section 4.1.5d).

The quality of data (e.g. sensitivity, reliability, repeatability) obtained from control animals and baseline measures has important bearing on the interpretation of results from animals treated with the test article (drug treated animals) (IPCS 1978). It is the influence that happiness as a construct of welfare and Refinement has on these dimensions of quality that is discussed below.

2.4.2 Sensitivity of measures and welfare related variation

Sensitivity describes the ability to reliably measure small changes (IPCS 2008; Table 2.2). It can be applied in regulatory toxicology at a number of levels (Table 2.2), but I shall focus on the ability to detect small changes in the outcome variable (e.g. measures in the core battery; Section 1.5.3) with chronic dosing of test article, to determine the effects if any, on the animals' biological systems. The sensitivity of a measure is constrained by the upper and lower limits of the biological response, the initial pretesting (baseline) levels and the ability to distinguish the response from background noise (variation). Sensitivity therefore is both an inherent property of the individual biological variable in the core battery and the method, which includes properties of the animals themselves (e.g. welfare state), as well as the way in which the measurement is made.

Ceiling and floor effects are known biological phenomena that constrain the limits of a response (see below) to stimuli and ultimately the threshold of change in biological outcomes that can be detected (e.g. ultimate sensitivity). They describe the upper and lower limits, whereby the biological variable cannot exceed (ceiling) or fall below (floor) a certain level in response to a stimulus or test article (Kjellström & Grandjean 2007).

The law of initial values, first coined in 1931, by Wilder (Wilder 1976), describes observations that the size of a physiological response to an external stimulus is dependent upon the initial (baseline) level. In general, the higher the baseline level the smaller the increase, but the larger the decrease in response to stimulation and vice versa. Therefore increased or decreased baseline levels may

constrain further incremental or decremental responses with testing (or dosing) (Jamieson 1993). This results in a negative correlation between the amount of change and the initial baseline level. The response is constrained in the first instance by mechanisms controlling homeostasis, which sets the absolute maximum and minimum values of the internal body environment.

However, a negative correlation can be produced with measurement error, whereby the dependent measure lowers the ceiling or heightens the floor effect, resulting in the clustering of responses around extreme values, thereby obscuring genuine differences (Martin & Bateson 2007). This can be a function of lack of sensitivity in the measurement itself, or induced if reactivity to the experimental stimulus results in higher or lower baseline levels (reviewed in Jamieson 1993; e.g. stress, poor welfare). A second feature of this response is that as biological parameters approach floors or ceilings they become less variable, making it more difficult to distinguish between and within individuals in response to testing. However, the law of initial values does not universally hold for all physiological measures (reviewed by Berntson *et al* 1994) and should be considered a guiding principle rather than a law because of the many inconsistencies that have been demonstrated since it was originally proposed (Jamieson 1993). Having said that, physiological responses under autonomic control, particularly cardiovascular measures (e.g. heart rate and blood pressure; Appendix 1.2), do seem to follow this principle (Berntson *et al* 1994). The relationship between welfare, Refinement and quality of cardiovascular measures are reviewed in Chapter 5.

Capture, handling and restraint are stressful for primates (Chapters 3, 4 & 5), resulting in high baseline heart rates and blood pressure levels displaying reduced variation between and within animals (Chapter 5). These stressors can result in a ceiling effect, reducing the sensitivity of ECG and high definition oscillometry methods in quantifying the magnitude of change with dosing, and hampering toxicologists' ability to establish a dose-dependent response - a critical component in safety and efficacy testing prior to human exposure. The resultant stress response produced by handling and restraint confounds our ability to quantify the magnitude and direction of toxicity induced changes on heart rate and blood pressure. With Refinement to handling and restraint, however, we observe a reversal of this trend, reducing the confounding effects and enhancing the

sensitivity of cardiovascular measures. As a consequence of improved sensitivity, through the removal of ceiling effects, we observed greater variation between animals (Chapter 5).

2.4.3 Control of variation to enhance reliability and repeatability

All animal models are subject to biological variation as a result of genetic and non-genetic factors and the complex interactions between them (Festing 2002; Howard 2002; See Figure 4.1.1 for examples of variation in blood parameters for macaques). This puts laboratory animal scientists at a distinct disadvantage compared to those working in traditional chemical and physical disciplines (Howard 2002). Whilst good experimental design aims to control this variation so as not to obscure any treatment effect (Festing 2002; Howard 2002), this is easier said than done. There are so many environmental influences that can either bias the results or contribute so much noise (background variation) that it's a complex process identifying the effects of any experimental treatment (Festing 2002; Howard 2002; Chapter 4). Variability can be introduced by the experimenter deliberately (wanted) and inadvertently (undesirable); the way in which known variables are incorporated into the experimental design, how the animal is prepared, experimental conditions under which the variable is measured and the precision with which the measurements are made all contribute to variability in data. Whilst some of these may be easily identified and steps taken to control them, the difficulty still remains that variation arising from the interaction between the animal and its environment are the most difficult to control, predict and measure, and indeed they may go unnoticed and limit our ability to interpret findings (Howard 2002; Chapter 4).

Relatively subtle variations in the way animals are kept, looked after, handled, restrained and dosed can have a major influence of behaviour and physiology of animals (Chapters 4 & 5). Animal-specific variation such as age, sex, strain, breed and origin are controlled by choosing a uniform population (see discussion below that this may be to the detriment of external validity). In the case of cynomolgus macaques used for regulatory toxicology this may not be straight forward due to animal availability (Chapter 4; discussed in Buchanan-Smith 2006). However, randomization when allocating animals to concurrent and treatment groups is used in an attempt to overcome this bias (Weekley *et al* 2002; Gad 2007). Approaches to control variation are often input-based (Buchanan-Smith 2006), concentrating on the steps required to get to the desired endpoint (e.g.

standardization, acclimatisation and habituation to the laboratory environment), often without a full understanding of what the endpoint should be (e.g. relaxed animals, in a stable state). This focus on inputs is not limited to laboratory animal scientists; such an approach has been adopted by welfare scientists conducting on-site welfare assessments (reviewed in Chapter 3).

2.4.3a Standardization

The process of standardization is advised by textbooks on laboratory animal science and in international guidelines on study conduct (e.g. ICH). The term standardization, however, has been applied in various ways: (a) the setting of standards; (b) defining properties of the animal and its environment (Beynen *et al* 2001); and finally (c) keeping experimental conditions the same for all animals (within-experiment standardization) or all experiments (between-experiment standardization) (Wurbel 2002; Buchanan-Smith 2006).

The setting of standards (a) has been applied across a multitude of levels (e.g. legislation, codes of practice, study conduct, quality assurance schemes), and involves prescribing minimum characteristics that should be met, including housing, husbandry, battery of biological variables to be recorded, timing and order of conduct of preclinical studies. The aim of such standards is to get to a desired endpoint such as the acceptability of study to regulatory bodies (e.g. OECD; US FDA) and enhanced welfare (see Buchanan-Smith *et al* 2004; Buchanan-Smith 2006 on the limitations of this approach to meet needs of animals), but does not necessarily lead to standardization in the true meaning (Buchanan-Smith 2006).

Of the remaining interpretations, one method currently growing in popularity adopts a 'list everything' approach (b) whilst the remaining (c) emphasizes environmental standardization; the 'standardize everything' approach, tries to equate testing apparatus, experimental protocols and all possible features of animal husbandry (discussed in Wurbel 2002; van der Staay & Steckler 2002). A major shortcoming in trying to standardize everything is that seemingly minor environmental differences can cause fundamentally different outcomes of the same experiment among different labs or independent replicates within labs; this is despite the extensive efforts of researchers to keep everything the same (Wurbel 2002). For example, a study by Crabbe *et al* (1999) seriously

questioned the efficacy of environmental standardization. Even though extraordinary lengths had been taken to use analogous testing apparatus, protocols, and approaches to keeping animals in three testing laboratories, there were significant differences in behaviour of mice of the same strain recorded across participating sites. This study clearly revealed that it is practically impossible to standardize everything and guarantee reproducibility of results across experiments. It was perhaps somewhat naïve or showed a lack of understanding of the complex interactions animals have with their environment, but not surprising when historically animals were considered simplified biological systems akin to cell cultures (e.g. Helma 2005).

Indeed humans are one of the most salient features of the laboratory environment (present as care staff, technicians, researchers; reviewed in Chapter 5). It is known that many commonly used species in the laboratory including primates (reviewed in Rennie & Buchanan-Smith 2006a) can discriminate between their human care takers, and the differential relationship can lead to profound changes in biological parameters (Chapter 5). Further the intraspecific social environment is often overlooked; primates have complex social lives, which it is impossible to standardize! Indeed the differential physiology induced by effects of a macaque's position in the social hierarchy have been used to study the effects of chronic social stress on cardiovascular parameters, atherosclerosis and heart disease (e.g. Shively *et al* 1997; Chapters 3 & 5).

Wurbel (2002) has argued against standardization for the sake of external validity. By limiting the effects of a given variable to a certain population of animals under specified conditions the generalizability of findings to other conditions, populations or species is severely restricted (Johnson & Besselsen 2002; Wurbel 2002; Buchanan-Smith 2006) - yet extrapolation of findings (Vissers *et al* 2001) is a key component in the identification of risk of adverse effects in humans and the main reason for conducting preclinical testing. Furthermore, there's a complex interplay between Reduction and Refinement, two of the 3Rs (see de Boo *et al* 2005 for discussion). For example changes that are positive for welfare (Refinement) may have a negative effect on Reduction (Buchanan-Smith 2006). Provision of environmental enrichment to enhance welfare has been a hotly debated topic in laboratory forums (see FELASA 2006; Hubrecht 2010 for discussion). For primates in laboratories the forms of enrichment are diverse (e.g. Lutz & Novak 2005; Honess

& Marin 2006), and whilst often reported to be beneficial for welfare (Buchanan-Smith 2010), it has wide-reaching effects on behavioural and physiological parameters and thus the potential for increasing variance in experimental outcomes and the number of animals required to identify a treatment effect (Mering *et al* 2001; Bayne 2005; FELASA 2006). Studies examining the effect of provision of environmental enrichment on variation of experimental outcomes in primates have not been reported (Buchanan-Smith 2006). However, that environmental enrichment increases variation (Buchanan-Smith 2010), does not necessarily hold true, at least when comprehensively reviewed for non-rodent species. Wolfer *et al* (2004) found that the performance of female mice housed in enriched home cages, in a range of behavioural tests did not show increased variation. This finding is supported by Garner (2005) who concludes that in barren housing the higher prevalence of abnormal stereotypic behaviour patterns is likely to compromise validity and reproducibility of results, therefore enriched housing should be the norm. Indeed, beneficial environmental enrichment should result in animals that are more like a 'naturally normal' animal in terms of their development, behaviour and physiology because these environments are closer to the environments in which animals have evolved (Section 2.2.2) and this in turn should lead to better experimental models with improved external validity (Garner 2005). Nevertheless further work is needed to clarify the relationship between housing conditions, external validity and welfare (Wurbel & Garner 2007).

2.4.3b Acclimatisation and habituation to the laboratory environment

Transportation and relocation of animals to the laboratory (Figure 1.4) for research purposes induces enduring physiological and behavioural changes. These changes are a departure from normal (compared to pre-transport levels; Section 4.1.5d). But given enough time animals adapt to the new environment, overcoming the stress of transportation and the effect of novelty: their physiological and behavioural variables normalize and show greater stability (Section 4.1.5d). This is known as acclimatisation[2] and it involves learning processes (e.g. habituation, desensitisation, socialisation; Chapter 5) by which the animal becomes accustomed to the new environment (and sometimes involves becoming familiar to testing protocols) prior to onset of study. This period of

[2] Acclimatisation is the term used to describe adaptation to a new climate or environment (JWGR 2009).

adjustment is critical to: (a) improving the sensitivity of biological measures (through the reduction of floor and ceiling effects observed with decreased stress response and improved welfare; Section 2.4.3), (b) enhancing the repeatability of measures because the animal is in a more stable state and (c) the reliability is likely to be greater if baseline measures are recorded once the animals behaviour and physiology have normalized. Although acclimatisation is recognised as critical for improving the quality of scientific outcomes (e.g. sensitivity, reliability, repeatability) and universally recommended (e.g. ICH), there's no defined period of acclimatisation, due to lack of scientific data that directly addresses the issue of precisely how much time should elapse before research begins (Capitanio *et al* 2006). Nonetheless, Capitanio *et al* (2006) having reviewed published studies conducted for other reasons, recommend that a period of three months should elapse from arrival before onset of experimental protocols (Section 4.1.5d). It should be noted that different biological parameters normalize and stabilize at different rates (Hassimoto *et al* 2004; Section 4.1.5d).

The process of acclimatisation is more often than not a passive process on the part of laboratory personnel; with the passage of time, it's assumed that animals get used to experiencing husbandry events as they happen, principally by habituation[3]. There's usually little consideration about what the animal is learning about the experience (see Chapter 5). This is different to the process of Refinement. When Refining husbandry and scientific procedures (e.g. Chapter 5), the same (and additional) learning processes (e.g. habituation and more proactive approaches including desensitisation and positive reinforcement training) are actively employed to ensure the animal associates something positive with various aspects of the laboratory environment. This is often undertaken by laboratory personnel in a more structured goal-orientated way. As a consequence, these types of Refinement have a greater effect upon welfare and quality of scientific outcomes than acclimatisation alone.

For the remainder of the thesis I will use the framework of terms defined in this Chapter, that are useful in measuring quality of scientific output, to examine the link between Refinement, animal welfare and scientific outcomes in cynomolgus macaques used for regulatory toxicology. Further,

[3] Habituation refers to the waning of a response over time (JWGR 2009).

using concepts outlined by Poole (1997) and his operational definition of happy, in Chapter 3 I have identified behavioural, physiological and physical health correlates in cynomolgus macaques. Assuming that happiness is necessary for good welfare, I can begin to examine whether unhappiness is a confounding variable that results in increased variation in biological parameters recorded from macaques.

2.5 Aims of the thesis

The link between good welfare and good science is often made but has not been systematically examined in cynomolgus macaques used for regulatory toxicology. They are a valuable model used to predict the safety of new drugs prior to human exposure. Macaques may suffer as a result of restrictive laboratory environments and harms caused by toxicological procedures. Suffering is at odds with good science; it alters behavioural and physiological parameters, introducing unwanted variation into experimental outcomes and may confound our ability to quantify drug-related changes in biological parameters. It is critical for both human safety and of ethical importance that their use results in good quality scientific output. The overarching aim of the thesis is to examine the link between welfare and quality of scientific output through Refinements to macaque use in regulatory toxicology. The term quality of science has a number of meanings, an important first goal therefore was to clarify terminology associated with quality and to narrow the concept to fundamental aspects of scientific output, and those most appropriate to toxicology include sensitivity and variation (reliability and repeatability) of measures in the core battery (this Chapter). A second aim is to identify feasible indicators of positive and negative welfare in cynomolgus macaques housed at the CASE sponsor by integrating approaches to welfare and adopting a multidisciplinary assessment protocol (Chapter 3). My final aim is to examine the effects of Refinements to the holistic environment of macaques used for regulatory toxicology (Chapters 4 & 5). Applying the defining characteristics of scientific output and welfare indicators, the link was examined using data mining techniques to evaluate longitudinal changes in housing and husbandry (Chapter 4), and the effects of Refinement of macaque-care staff contact were examined using a between subjects design. I was specifically interested in whether enhanced welfare (a *priori* condition for Refinement) lead to measurable improvements in sensitivity of individual biological variables, through more normal baseline values (e.g. via the removal of ceiling or floor effects), and

whether enhanced reliability and repeatability of toxicological measures was observed by reducing the variation within and between animals.

3

Assessment of welfare of cynomolgus macaques in regulatory toxicology

"*KRA of the Malays.*"

"*The Malay name has frequently a close resemblance to the cry of the animal it designates (Kra); and this is remarkably the case in the present instance…*"

Raffles (1821), p 247.

*"Early accounts of the behaviour of long-tailed macaques (*M. fascicularis*) emphasized their "loquaciousness" and postulated that they are both more vocal and possess a more "extensive vocabulary" than their congeneric relatives."*

Palombit (1992 a), p 144.

"I like to listen. I have learned a great deal from listening carefully. Most people never listen."

Ernest Hemingway.

Abstract

*The assessment of macaque welfare is fundamental to Refinement and necessary to bring about improvements in care, housing and the conduct of procedures. Examples of indicators used to monitor the welfare of laboratory-housed animals have been proposed in recent reviews (e.g. JWGR 2009; 2011). However, a coherent framework specific to cynomolgus macaques (*Macaca fascicularis*) used in regulatory toxicology has not been published. Moreover information on species-specific indicators, particularly behavioural, cardiovascular and clinical parameters, are dispersed in divergent publications (e.g. primate, laboratory animal, toxicology and veterinary literature), and they are not easily accessible to care staff or even those researchers wishing to study welfare in the laboratory. There's a need to collate and summarise what is currently known about species-specific indicators, so the introduction to this Chapter is dedicated to that aim. Moreover, indicators must be (a) feasible for use under the constraints of laboratory conditions, (b) valid - reflect the phenomena being measured, (c) sensitive enough to reveal changes in welfare state and ideally what's causing them, and (d) reliable such that we get the same result each time we measure it under the same conditions by the same and different persons responsible for animal care. The main aim of this Chapter was to examine the validity, sensitivity and reliability of each parameter before integration into an overall assessment of welfare of cynomolgus macaques used for regulatory toxicology. The final welfare assessment framework can be used to detect changes in welfare status of macaques in response to planned manipulations (Chapter 5).*

3.1 Introduction

Refinement is a vital component of humane (Section 1.2) and good science (Chapter 2). It is enshrined in legislation and codes of practice and is the formal framework by which animal welfare is considered in the laboratory (Section 2.1). Successful Refinement depends upon the ability to effectively assess animal welfare (JWGR 2011) as indeed it is ethically incumbent on humans who care for animals to do so (Chapter 2). In the laboratory, this responsibility lies with a number of staff, for example husbandry technicians, licensees, Named Animal Care and Welfare Officers (NACWO) (collectively known as 'care staff' throughout the thesis) and (Named) veterinarians. Care staff are tasked with ensuring the welfare of individual animals in response to the laboratory environment, husbandry and scientific procedures as part of their daily duties. Indeed current unit procedures in the host laboratory are comprehensive; regular, numerous daily checks of animal and environmental variables are made, and more detailed weekly health assessments are performed (Chapter 5). In addition the demands of study may require staff to monitor different indicators more frequently depending on the likely effects of the test article (e.g. neurobehavioral function assessment; Gauvin & Baird 2008; Moscardo *et al* 2010).

In Chapter 2, I discussed the concepts of animal welfare, approaches to assessment and limitations in their application. Welfare is multidimensional and therefore can be most adequately assessed through a number of measures, each linked to a specific (or multi) dimension (s) of welfare (Botreau *et al* 2007). In addition, two types of measures have been employed in welfare assessments; those measuring aspects of the animals' environment (e.g. termed 'input' measures – stocking density, aspects of husbandry etc.) and those measuring attributes of the animals themselves, as indicators of how they are faring in those environments (e.g. 'output' measures - health, behaviour, physiology) (Webster *et al* 2003; Whay *et al* 2003a). Animal-based measures are generally considered to be more closely linked to the welfare of animals, indeed welfare itself is a property of the individual animal (Capdeville & Veissier 2001; Whay *et al* 2003b).

The selection of animal-based indicators, for use *in-situ,* is based upon them meeting the same fundamental aspects of quality that I used to examine scientific output (Table 2.2; Chapter 2). Namely, they must be fit for purpose; as evidenced by meeting a framework of demands including

validity (e.g. reflect welfare), sensitivity (e.g. reveal amount and direction of change in welfare state), reliability and repeatability (e.g. closeness of agreement each time we measure welfare under the same conditions by the same and different persons responsible for animal care). In addition to meeting these demands, welfare assessment protocols for animals held in laboratories (and also zoos, kennels, farms etc.) place additional demands on the measures – they should be feasible, practicable for observation and measurement in a busy commercial environment.

Despite being the most commonly used nonhuman primate for research and testing in Great Britain, and the large amounts of biological data generated during toxicology studies, there are few data published about species-specific welfare indicators. Further, what is known is often spread through divergent publications, making it inaccessible for care staff. There's a need therefore, to collate and summarise current knowledge of assessment of welfare in the cynomolgus macaque into a framework for practical use in primate units. This will also aid in identifying those measures that are feasible and valid before testing their fitness for purpose in the laboratory. This was my first aim.

A good starting point for this study, which aims to integrate multiple measures (Novak & Suomi 1988; Suomi & Novak 1991) into an overall assessment of welfare (Botreau *et al* 2007), was to build upon current unit procedures and those measures, recorded at baseline in macaques, that aren't traditionally used by care staff to reflect welfare, but are considered useful parameters. Examples include physical health (e.g. signs of disease, physical abnormality, body damage, fluctuations in body weight etc.) and physiological parameters such as clinical pathology (haematology and clinical chemistry; Appendix 1.1) and cardiovascular (heart rate and blood pressure; Appendix 1.3 – 1.5) measures recorded as part of the core battery (Section 1.5.3; Appendix 1.1 – 1.5). The sensitivity, reliability and repeatability of these parameters have rarely been examined with animal welfare in mind. A second aim of this Chapter therefore, was to assess the fitness of use of these measures as indicators of welfare state in macaques.

In addition to those parameters listed above, macaque behaviour is observed by care staff but recorded in a less formal manner. A final aim of this Chapter was to link behavioural parameters

with physical health and underlying physiological changes in macaques. This is perhaps the most critical of aims, because behaviour is one of the most visible and accessible of all the selected welfare indicators for care staff to monitor, but it is commonly misinterpreted (JWGR 2009).

3.1.1 Indicators of welfare in the cynomolgus macaques

Behavioural, physiological and physical health measures can be recorded to determine welfare (Chapter 2). These parameters normally vary within certain limits, but more extreme variations out with the range of normal fluctuations are indicative of changes in welfare. In order to infer welfare from changes in responses, experimental manipulations may be deliberate on the part of the researcher or piggy backing on husbandry or experimental events where other parameters show them to be negative or positive experiences for animals. A change in welfare may be inferred by comparing responses before and after changes (with animals acting as their own controls) or between controls (not subject to the manipulation) and treated animals. Moreover, pharmacological and neurological studies that artificially induced changes (e.g. anxiety, physiological stress; Ninan *et al* 1982; Schino *et al* 1996; Kaplan *et al* 2009; Shively *et al* 2009) have also been reviewed.

In Chapter 2, I argued that for good quality toxicological output, animals must be in a normal, stable, healthy and happy state, as an inherent quality for good welfare. Poole (1997) provides an operational definition of happiness and a framework of behavioural measures with which to assess it (Chapter 2). I will start my review of putative indicators of welfare in cynomolgus macaques by examining which behaviours vary with changes in welfare.

a) Behaviour

The expression of species-typical behaviour is highly variable between animals, and a good knowledge of the normal range and frequencies of behavioural responses is a prerequisite to infer improved or reduced welfare from observed changes (Olsson *et al* 2003). Whilst many studies have attempted to identify behaviours associated with subjective negative feelings, there are considerably fewer studies aimed at identifying behavioural indicators associated with positive affective states (Table 2.1; Chapter 2). Poole (1997) lists the behavioural characteristics that he

believes to be indicative of a happy animal: display a wide behavioural repertoire - alert and busy, able to rest in relaxed manner, confident – outgoing, not showing fear to non-threatening stimuli and not displaying abnormal behaviour. I will explore the types of behavioural responses that meet these characteristics below.

Normal activity budgets

Cynomolgus macaques have evolved to live in large groups; in the wild they're diurnal, spending the majority of the day engaged in social behaviours, foraging for food, and follow distinct patterns of alternating activity/rest, whilst at night they roost in trees (Table 4.1.1). This organisation of activity and naturalistic pattern can serve as useful benchmarks for improving the quality of life of captive macaques (Lindburg 1991; Chapter 2).

In the laboratory, changes in activity budgets have been observed following stressful events such as room and cage change (Crockett *et al* 1995), social regrouping (Shively *et al* 1997) and air transport/relocation to a new facility (Honess *et al* 2004; Brent & Veira 2002). For example, Crockett *et al* (1990; 1993; 1995) found that singly housed cynomolgus macaques showed disrupted sleep patterns, with a significant reduction in sleep before midnight, and reduced diurnal activity, and food consumption on the day after the room and cage change. These behaviours were accompanied by elevated urinary cortisol, indicating macaques found the events stressful (for discussion on disrupted sleep and welfare see below). Nonetheless baseline recording of behaviour found the proportion of time spent inactive (50%) during the day to be relatively high. This finding should be cause for concern as it is higher than those reported for animals in the wild (e.g. 33-37% resting, males and females; van Noordwijk *et al* 1993) and for group housed animals in laboratories (e.g. Honess *et al* 2004). Furthermore, single housing is inadequate for meeting the social and behavioural needs of macaques and is widely considered to be detrimental to psychological wellbeing (Chapter 4).

Honess *et al* (2004) found a significant shift in the activity patterns of cynomolgus macaques following air transport and relocation. They observed a reduction in the amount of time spent locomoting and an increase in duration of 'hugging' immediately after arrival when compared to

pre-transport observations at the breeding facility. Furthermore, three weeks after arrival, behaviour patterns had not returned to baseline levels, but the duration of play had increased, whilst allo-grooming, important for maintaining social bonds (see below) had decreased. Transport and relocation to a new facility are stressful and activate the hypothalamic-pituitary-adrenal axis (HPA; Chapter 4). Similarly, Brent and Veira (2002), found 'contact' (passively touching), to increase initially following translocation and regrouping and remain high for the first three weeks, but decreasing over time with the 13 week acclimatisation period. They also found allo-grooming, reached its peak three weeks after arrival, followed by a drop and levelling off as animals became more accustomed to their social group and the new facility.

Long-term sleep disruption may also have important welfare implications for macaques housed in laboratories, particularly if sleep is disturbed following common husbandry events (e.g. room and cage change; Crockett *et al* 1990; 1993; 1995). Although not studied specifically in macaques, sleep deprivation has detrimental effects on physiological and psychological functions in humans and rats. For instance, in laboratory rats, chronic sleep deprivation is associated with behavioural changes, impaired cognitive ability, and altered metabolic activity including poor thermoregulation (reviewed by Everson 1995). In addition, cerebral and endocrine functions are also disrupted, resulting in an impaired immune system, and increases the risk of morbidity and mortality. In humans too, sleep deprivation has a negative impact on metabolic and physiological parameters, including tissue repair, immune system regulation and thermoregulation (Banks & Dinges 2007; Walker 2008). Although, the specific effects of sleep deprivation on cellular and humoral immunity remain largely unexplored, a few studies have found evidence of reduced antibody production following immune challenge (e.g. vaccination; Banks & Dinges 2007). Furthermore, the psychological and cognitive effects of sleep deprivation (e.g. depression, poor memory, reduced behavioural alertness and problem solving, and decision making skills) can be equally impairing (see Banks & Dinges 2007; Killgore *et al* 2008). More recently, sleep researchers have begun to explore the associations between sleep disruption and altered cardiovascular function in humans (Sforza *et al* 2004). Sforza and colleagues (2004) found patients experiencing disrupted sleep had raised heart rates and blood pressures – both of which are associated with an increased risk of developing cardiovascular disease. Thus chronic sleep disruption has potential negative

consequences for macaque welfare, and may also alter some of the biological variables (e.g. haematological and cardiovascular parameters) of interest to toxicologists during preclinical safety testing.

A common feature in the studies given as examples above was the shift in the amount of time spent engaged in social behaviours, affiliative behaviours in particular. The significance of the performance of these types of behaviours to welfare are reviewed below.

Affiliative behaviour

Affiliative behaviour is an umbrella term that describes social, body contact that is non-aggressive and non-assertive in nature (Das *et al* 1998), examples include social grooming, touching, embracing and sitting in contact with another (Aureli *et al* 1989; Das *et al* 1998). Social grooming (allo-grooming) is considered important in the development and maintenance of social bonds and group cohesion in primates (Schino *et al* 1988; Dunbar 1991) and it may reflect a strong friendly relationship between participants or act as a behavioural mechanism for reduction of tension (Schino *et al* 1988; Aureli *et al* 1989; Boccia *et al* 1989; Aureli & van Schaik 1991; Troisi *et al* 1991).

Its performance is associated with a number of physiological changes. For example, in pigtailed macaques (*Macaca nemestrina*), receiving grooming is linked with a reduction in heart rate (Boccia *et al* 1989), and following social separation, its performance stimulates an increased release of endogenous opioids in talapoin monkeys (*Miopithecus talapoin*; Keverne *et al* 1989). Both high levels of grooming and opioids were thought to have a calming effect on monkeys following the stress of social separation. Furthermore, levels of affiliative behaviour observed following conflict, in cynomolgus macaques, are associated with a faster decrease in rates of displacement behaviours (e.g. scratching, body shake etc., see below; Aureli *et al* 1989; Das *et al* 1998).

Indeed, Schino *et al* (1990) found an inverse relationship between the quantitative differences in grooming exchanged between partners and the level of conflict/tension (Schino *et al* 1990). Furthermore, the authors also found that the frequency of displacement activities continued to increase over time until the onset of grooming. Therefore increased frequency and duration of

bouts of grooming are likely indicators of high stress, but also that the animal is employing a behavioural mechanism (e.g. allo-grooming) to cope with it (Schino *et al* 1990).

This coping mechanism has been found to have protective effects on monkeys' immune systems in the laboratory. For example, in male cynomolgus macaques subjected to successive regrouping, higher levels of affiliative behaviours were associated with the least diminished immune response (Cohen *et al* 1992; Line *et al* 1996). Moreover Capitanio and colleagues (1998 a, c) demonstrated a positive correlation between levels of affiliation and survival after SIV infection in rhesus macaques. Therefore performance of affiliative behaviours temporarily protected monkeys from the effects of serious viral infection (Schapiro *et al* 2000a; 2001). This ameliorative effect may also be referred to as social buffering, whereby a social partner lessens the response to stressful events (Kikusui *et al* 2006; Gilbert & Baker 2011; Chapter 4) and it has been shown to benefit the health and welfare of primates in laboratories (Gust *et al* 1994; 1996; Schapiro & Bushong 1994; Schapiro *et al* 1996; Chapter 4). Moreover, social housing is enriching, increasing the opportunities for species typical behaviour and social play (Line *et al* 1990; 1991; Schapiro *et al* 1996).

Play behaviour

Playing has been found to be the most frequent social activity recorded in juvenile and infant cynomolgus macaques (Md-Zain *et al* 2010), with males typically playing more than females (van Noordwijk *et al* 1993; Md-Zain *et al* 2010). It is accompanied by characteristic facial expressions, gestures and postures (see below). Although play behaviour mainly occurs in juvenile animals (Fagen 1981), it varies quantitatively and qualitatively within and between individuals of a given species (Poirier & Smith 1974). Play has long been identified as a potential welfare indicator (e.g. Fagen 1981; Lawrence 1987), because it often disappears when animals are under fitness challenges (Held & Spinka 2011), for example when they are sick, hungry, under thermal stress, at risk of predation etc. (Martin & Caro 1985). However, animal play may also arise during socially stressful situations, such as immediately before feeding in captive bonobos (*Pan paniscus;* Palagi *et al* 2006) or when an unfamiliar male is being integrated into a group of resident sifaka (*Propithecus verreauxi*; Antonacci *et al* 2010). Animals may therefore use play interactions to reduce social tensions (Held & Spinka 2011). Furthermore, increased frequencies of play are observed following

challenging conditions, indicating a rebound effect in response to new opportunities for play (e.g. piglets transferred from barren pens, Wood-Gush *et al* 1990; calves released after a period of confinement, Jensen 1999). The presence of play or its increase may indicate improving welfare after a period of social stress or deprivation, rather than good welfare in its own right (Held & Spinka 2011).

However, play is often linked to positive emotions (Barnard 2004; Burgdorf & Panksepp 2006) and is in itself rewarding (reviewed in Held & Spinka 2011). Play is typically spontaneous, often appearing independent of external factors (Burghardt 2005) and it has been used in laboratory studies as a powerful reinforcer for conditioning animals to experimental, operant paradigms, ranking above other rewards such as food or petting (Humphreys & Einon 1981). Social play therefore, is something the animal will work for, indicating it is rewarding in its own right (Rolls 2005). Increases in play fighting, however, may be accompanied by negative affect caused by social stress particularly in hierarchical animals, as it can be used as a way of asserting dominance (Mendl *et al* 2010). Thus play fighting does not always indicate that animals are experiencing pleasure and good welfare (Held & Spinka 2011).

Nonetheless, despite these notes of caution, the expression of play more often than not indicates the absence of poor welfare and the quality and quantity may reflect the social, physical and emotional functioning of animals (Fagen 1981; Lawrence 1987). It has potential therefore as an indicator of positive welfare in animals (Held & Spinka 2011).

Facial expressions

As described above in relation to play behaviour, macaques have evolved facial expressions, vocalisations and postures as means of communicating with one another and managing social interactions (Hinde & Rowell 1962; van Hooff 1967). The facial expressions of the *Macaca* genus have been widely studied and well characterised (e.g. van Hooff 1967; Redican 1975). Macaques possess a large range of facial movements, displayed during aggression, tension, fear, submission, alertness, relaxation and play (van Hooff 1967; Preuschoft *et al* 1995). They are considered to reliably indicate the underlying emotional state of the individual (Preuschoft 1995; Zeller 1996).

However, they have not been widely used as measures of welfare, nor have they been correlated with other indicators, yet they may be useful for assessing animals' reactions to approach and handling or restraint by care staff (JWGR 2009; this Chapter; Chapter 5).

For example, rhesus monkeys restrained in a chair and threatened with capture by a human experimenter (e.g. directly staring at monkey and approach with capture net) directed high levels of fearful facial expressions towards the threat (Nakayama *et al* 2005). Chair restraint is known to be physiologically stressful for monkeys leading to pronounced activation of the HPA (Kling & Orbach 1963; Mason 1972; Mason *et al* 1973; Nakamura *et al* 1992; Norman & Smith 1992; Morrow-Tesch *et al* 1993; Norman *et al* 1994). In an experiment to validate nasal temperature as a measure of stress, increased temperature immediately followed restraint in a chair and was accompanied by macaques' fearful responses (Nakayama *et al* 2005). Furthermore, Clarke and colleagues also found increased durations in fearful displays (including facial expressions such as grimacing) and screech/alarm vocalisations (see below) with stressful events. In successively, escalating stress tests (e.g. harness fitting, novel room, restraint board), fearful behaviours co-varied with other behaviours indicative of reactivity (e.g. struggling, increased locomotion, depressed posture) and positively correlated with heart rate and cortisol levels. As Clarke *et al* (1988) observed, facial expressions are often accompanied by vocalisations and distinct body postures.

Vocalisations

Cynomolgus macaques have an extensive vocal repertoire (Palombit 1992a, b) and early field studies describe them as one of the most "loquacious" of the macaque genus (reviewed in Palombit 1992a). Palombit (1992 a, b) categorised calls given by cynomolgus macaques recorded in the wild and found they co-varied with conditions, and the caller's arousal. For example macaques vocalised during affiliative contact, group movements, submissive displays, agonistic recruitment, dominance aggression, inter-group aggression, predator alert, and sexual behaviour (Palombit 1992 a, b).

Harsh calls are generally given by agitated animals, involved in aggressive encounters, under threat and by animals distressed during separation from group mates. They can be pronounced during

handling by human care staff (Heath 1989) and may therefore indicate reduced welfare. The distinction between clear "coo" calls is less clear cut (Levine *et al* 1987). Whilst they are given by animals when engaged in grooming and during feeding they are also emitted by animals seeking contact during brief periods of separation. To the human observer, coo calls given in these contexts may sound the same even though they have acoustically different properties when subjected to spectrographic analysis (Levine *et al* 1987; Palombit 1992a). This somewhat clouds their interpretation as a welfare indicator, as the observer may not be able to discriminate between positive (e.g. grooming and feeding) and negative events (e.g. separation from group mates) by listening to calls alone. Furthermore calls that are given in specific contexts in the wild may not be given in captivity. Yet, comparisons made between descriptions of vocalisations emitted in captive and wild pig-tailed macaque (*M. nemestrina*) populations complement one another's accounts (Grimm 1976; Caldecott 1986).

In captive situations changes in the frequency and occurrence of vocalisations were found to accompany changes in housing and treatment conditions (Mulligan *et al* 1994), and they have been used to assess the effectiveness of environmental enrichment (Crowell Comuzzie 1993; Boinski *et al* 1999). For example, brown capuchins (*Cebus apella*) were found to emit fewer alarm vocalisations in response to a threatening stimulus (human observer), when they were given physical environmental enrichment. Furthermore, the call rate was significantly positively correlated with levels of faecal cortisol and abnormal behaviour. Therefore the frequency of alarm calling was a useful indicator of stress in response to the presence of a threat, in conditions of low environmental enrichment.

The relationship between primate vocalisations and circulating plasma corticosteroids is less clear in very young animals (review by Levine *et al* 1987). Simply measuring the frequency of calls emitted during mother-infant separation is not sufficiently reliable for evaluating activation of the pituitary-adrenal system. With spectrographic analysis, however, researchers were better able to discriminate the quality of calls (Levine *et al* 1987). None the less in adult rhesus macaques, Bercovitch *et al* (1995) found by blocking glucocorticoid output following administration with an adrenal steroidogenic inhibitor, treated monkeys did not alarm call in response to threat of

capture, whereas untreated controls did. Moreover, they gave less harsh alarm calls compared to controls, when their offspring were being handled by a human carer, demonstrating a relationship between type and frequency of vocalisations and physiological stress response.

Postures

In addition to facial expression and vocalisations, body postures may also indicate the emotional arousal and physical wellbeing of macaques. Withdrawal postures such as crouching (pressing the body against the floor), hiding the head and ventral side, are associated with fear (de Waal *et al* 1976) whilst presenting rear with a silent-bared teeth display is indicative of submission (Hinde & Rowell 1962; de Waal *et al* 1976; Aureli *et al* 1989). These behaviours, although part of the normal behavioural repertoire, may be expressed towards humans in the laboratory, indicating anxiety about their proximity (JWGR 2009). Extreme variations of these postures are seen in behaviourally depressed, subordinate animals that have been subjected to repeated regrouping (Kaplan *et al* 1996). Accompanying the crouched postures were heightened states of vigilance (Shively *et al* 1997; Shively 1998) and elevated cortisol levels (Shively *et al* 1997; Shively 1998), indicating these animals are likely to be experiencing poor welfare.

Displacement activities

Displacement activities are considered as a direct behavioural expression of internal conflict (Hinde 1970); they are performed during times of anxiety (Troisi 2002) or heightened social tension (Easley *et al* 1987; Schino *et al* 1988; 1990; Aureli & van Schaik 1991) and may indicate reduced welfare (Maestripieri *et al* 1992; Troisi 2002). They can be induced by administration of anxiogenic (anxiety increasing) drugs (Schino *et al* 1996; Troisi 2002) and with electrical stimulation of areas of the brain associated with acute stress response (Redmond & Huang 1979; Ninan *et al* 1982). Amongst primates the most commonly reported are self-scratching, self-grooming, body shaking (Schino *et al* 1988; 1990; Aureli & van Schaik 1991; Maestripieri *et al* 1992), and yawning (Hinde & Rowell 1962; Rowell & Hinde 1963; Bertrand 1969; Schino *et al* 1988; Troisi *et al* 1991). Thus, if performed at high frequencies they may be considered to be an indicator of stress (Maestripieri *et al* 1992).

Abnormal behaviour

Stereotypies and abnormal behaviours are often used as indicators of diminished welfare in animals (Paulk *et al* 1977; Goosen 1981; Dawkins 1988; 2003; Bayne *et al* 1991 a; b; Lawrence & Rushen 1993). Their precise aetiology and function are unclear (Dantzer 1989; Mason 1991; Dantzer & Mittleman 1993; Rushen *et al* 1993). They are defined by their apparent lack of adaptive function and manifest as bizarre, repetitive and unvarying behavioural patterns (Mason 1991). Incidence, severity and form of these behaviours vary across species of primates (Novak & Bollen 2006) and in captive animals, their prevalence is higher in laboratories than in zoos (Bollen & Novak 2000). The types of bizarre behaviours that primates display include rocking, cage circling, flipping, pacing and swaying (Erwin *et al* 1973; Paulk *et al* 1977; Bryant *et al* 1988; Crockett *et al* 1995) along with other less well reported ones such as bar strumming (repetitive "strumming" of bars with fingers) and rubbing biscuits on the body (Crockett *et al* 1995).

These behaviours often occur in impoverished, unstimulating, suboptimal environments (Berkson 1968; Goosen 1981; Sackett *et al* 1981; Mason & Turner 1993; Mason 2000), although the threshold of performance denoting poor welfare is not clearly defined, and what is more, the occurrence of these behaviours may not be reflective of current welfare problems. Their performance can become emancipated from environmental stimuli and they may be performed independently of conditions that lead to their development (Mason & Latham 2004). Even so, it is generally considered that their occurrence should be viewed as a warning of animal suffering (Mason & Latham 2004).

With discussions of behavioural indicators I have already introduced some likely physiological parameters, which co-vary with behaviour, and may therefore be used to monitor welfare in the laboratory. Physiological indicators will be discussed in more detail below.

b) Physiology

One potential indicator of an animal's welfare is the presence or absence of stress (Moberg 2000; Section 2.2.4 discusses the link between stress and welfare). The experience of stress causes significant biological changes in the animal's endocrine, behavioural, autonomic nervous and

immunological systems. These endpoints have been used to measure stress (Moberg 2000; Shively & Willard 2012). Often the first response, in the case of many stressors, is a behavioural one (e.g. acting to avoid or remove the animal from the stressor). The next line of defence involves the autonomic nervous system – the basis of Cannon's (1929) 'flight-or-fight' response. The autonomic nervous system (ANS) affects cardiovascular (Appendix 1.2), gastrointestinal, exocrine and adrenal medulla activity (Moberg 2000). The biological results of this first response are often short-lived and therefore their value as indicators of long term welfare is minimal (Moberg 2000). In contrast to the effects of the autonomic nervous system, the endocrine system, or rather the hormones secreted from the hypothalamic-pituitary adrenal axis (HPA) can have a broad, long-lasting effect on the body including its immuno competence, reproduction and behavioural responses, which are regulated through pituitary hormones (Moberg 2000). A discussion of both these branches of the physiological response, as indicators of welfare, appears below.

Cardiovascular parameters (heart rate and blood pressure)

Many factors affect heart rate (e.g. body position, posture, size, age, sex, health, environmental temperature, activity level and emotional state; Chapter 5). Heart rate is often elevated in response to emotional stimuli and environmental stressors (Suomi *et al* 1981; Higley & Suomi 1989, Coelho *et al* 1991; Manuck *et al* 2009; Shively *et al* 2009), but it may be difficult to distinguish between the characteristics of the evoking stimuli (e.g. positive or negative; pleasure or fear arousing) as they produce similar cardiac responses. However, in properly controlled studies, changes of heart rate and blood pressure can be used in conjunction with other measures as evidence of changes in health and welfare (Manuck *et al* 2009; Shively *et al* 2009). Changes in these parameters as a result of arousal in response to handling, restraint and human interaction are reviewed in more detail in Chapter 5.

With repeated arousal and activation of the ANS, elevated heart rates and blood pressures correlate with behavioural and hormonal changes associated with HPA activation (Shively *et al* 1997; Kaplan *et al* 2009; Shively *et al* 2009). Variation in heart rate response to challenge has been found to differ as a consequence of psychosocial stress in macaques (Kaplan *et al* 2009; Shively *et al* 2009). Indeed this response has been well characterised in cynomolgus macaques used as a model for

coronary artery atherosclerosis and coronary heart disease (Shively *et al* 2009). The model exploits macaques' natural tendency to form stable, hierarchical, social groups and to be intolerant of intruders (Kaplan *et al* 2009; Shively *et al* 2009). Reorganising small groups of monkeys, thereby inducing social instability, creates differential levels of stress amongst individuals in the newly formed groups related to their rank in the hierarchy (Kaplan *et al* 2009). Both female subordinates and dominant males have been found to be more stressed than other members of the group as evidenced by their behavioural, endocrine, cardiac responses and the development of cardio-pathologies (Kaplan *et al* 2009; Shively *et al* 2009). For example dominant males were observed in more frequent contact aggression (Kaplan *et al* 1982; 1983; 1995) whereas subordinate females show extreme fearful avoidance reactions sometimes associated with depressive-like collapsed behavioural postures (Shively *et al* 1990; 1997; 2009).

For dominant males, stress results from continual challenge to and with defending of their position in the hierarchy (Kaplan *et al* 1982; 1986; Manuck *et al* 1983), whereas for subordinate females avoiding the aggression and harassment of others is stressful (Shively *et al* 1987; Kaplan1991). In both cases the experience of stress triggers significant, reliable and persistent cardiac responses mediated by continual activation of the sympathetic nervous system (Strawn *et al* 1991). These socially stressed animals share many characteristics (e.g. endocrine responses, pathological changes and susceptibility to cardiac disease; reviewed in Kaplan *et al* 2009; Shively *et al* 2009). When they experience standard challenge tests (e.g. exposure to a novel room or threat of capture) and even in the mere presence of the human experimenter during normal husbandry events (Manuck *et al* 1986; 1989), they characteristically produce rapid elevations in heart rates and blood pressure, that remain higher for longer than other members of the social group (Shively 1998; Strawn *et al* 1991). The differences in heart rates are pronounced, with socially stressed animals showing 40 bpm elevations above counterparts (reviewed in Kaplan *et al* 2009; Shively *et al* 2009).

Heart rate variability can be used to investigate the balance between sympathetic and parasympathetic activity as a more precise measure of functioning of the autonomic nervous system under stress (von Borell *et al* 2007). Heart rate variability is derived from the intervals

between consecutive heart beats (Moss 1995), which are characterised by irregular intervals, as a result of oscillation in the regulatory components of cardiac activity (e.g. branches of the sympathetic and parasympathetic nervous system; Appendix 1.2; Cerutti *et al* 1995; Akselrod 1995). Stress responses can be quantified by identifying the relative changes in parasympathetic activity (e.g. vagal tone) characterised by alterations in the irregular intervals between successive heart beats (von Borell *et al* 2007). For example, Bowers *et al* (1998) used a measure of heart rate variability in the respiratory frequency range, called respiratory sinus arrhythmia (RSA) as a more sensitive measure of stress compared to heart rate alone (Porges 1985). Under stress the sympathetic system becomes predominant and the parasympathetic system is inhibited, thereby decreasing or eliminating vagal influence (reviewed in von Borell *et al* 2007) on heart rate. Reactivity of cynomolgus macaques to two kinds of stressors; a sudden loud noise (whistle blow) and threat of capture by an unfamiliar technician, were determined from changes in heart rate and RSA. The variance of RSA was found to be more sensitive, it discriminated the reactive differences between animals experiencing the same stressor and between the two stressors. Heart rates however, were elevated similarly across animals and treatments. On the basis of changes in RSA, researchers found cynomolgus macaques to react more strongly to exposure to threat of capture than sudden loud noise. These data matched their behavioural observations which found macaques to be inconsistent in their responses to the sound of a whistle; some animals orientated to and approached the direction of noise to investigate whilst others were more fearful (Bowers *et al* 1998).

As consistently observed by Shively and colleagues, working with cynomolgus macaque models of disease, an endocrine response is observed with persistent arousal during periods of stress; these responses are discussed below.

Hypothalamo-Pituitary-Adrenal (HPA) axis

The basic mechanisms by which endocrine responses are mounted are well established, and the hypothalamo-pituitary-adrenal axis has been the primary neuroendocrine axis monitored (Moberg 2000; Shively & Willard 2012). Increases in the circulation of the adrenal glucocorticoids (e.g. cortisol) have been long equated with stress (Moberg 2000) in response to handling and restraint,

housing, social conditions, transport and relocation to a new facility etc. (e.g. Clarke 1991; van Schaik *et al* 1991; Crockett *et al* 1993; 1995; Clarke *et al* 1995; Kim *et al* 2005 b; Fernstrom *et al* 2008; Lee *et al* 2010; Shively & Willard 2012). Secretion of prolactin and growth hormone are proving equally sensitive to stress (e.g. van Schaik *et al* 1991; Botchin *et al* 1993; Shively *et al* 1995; Kaplan *et al* 2003). Likewise, thyroid stimulating hormone and gonadotrophins (e.g. luteinising hormone, and follicle stimulating hormone) are either directly or indirectly modulated by stress (Moberg 2000; Williams *et al* 2001; Stavisky *et al* 2001; Sandoval-Guzman *et al* 2004; Czoty *et al* 2009). These types of measures invariably require invasive monitoring (e.g. repeated blood sampling procedures) although increasingly some may be measured either directly or via their metabolites in other body fluids (e.g. saliva, urine, faeces or hair; van Schaik *et al* 1991; Crockett *et al* 1993; Clarke *et al* 1995; Stavisky *et al* 2001; Bahr *et al* 2000; Mohle *et al* 2002; Heistermann *et al* 2006; 2010; Paramastri *et al* 2007; Hau & Royo 2009; Girard-Buttoz *et al* 2009). However, additional physiological recording beyond those required for baseline measurements by the CASE sponsor were not possible in this study. I will therefore not expand further on these measures, other than to highlight that alteration in biological function as indicated by changes in behaviour, physical health, cardiac responses and circulating clinical pathology analytes may be seen in response to changes in the pattern of release of these hormones when the animal mounts a stress response (e.g. neutrophil/lymphocyte ratio follows a similar pattern of change with increased cortisol level; Kim *et al* 2005a).

Immune parameters

Suffering from stress has long been attributed to increased incidence of disease in animals (Moberg 2000) due to suppression of immune competence (Cohen *et al* 1997; Capitanio *et al* 1998a; 1999; 2008; Shively 2012). Measures of immune function include alterations in circulating antibody titres (Scanlan *et al* 1987; Laudenslager *et al* 1993; 1999; Helms *et al* 2012), cell-mediated responses (Line *et al* 1996; Schapiro *et al* 2000 a; 2002) and other haematological variables which play a role in mounting an immune response (e.g. Leukocytes and their differentials: Neutrophils, Lymphocytes, Monoctyes, Eosinophils, Basophils; Appendix 1.1). An extensive range of physical and psychological stressors produce rapid responses in leukocytes that can affect the immune system's ability to cope with potential challenge (Loomis *et al* 1980; Yoshida *et al* 1986b; Ellard *et*

al 2001; Honess *et al* 2005 b; Kagira *et al* 2007). The relative changes depend upon the nature and intensity of stress (McLaren *et al* 2003) and a characteristic leukogram (ratio of neutrophils : lymphocytes) accompanies elevations in cortisol (Ives & Dacks 1956; Goosen *et al* 1984; Morrow-Tesch *et al* 1993; Capitanio *et al* 1998 b; Winterborn *et al* 2007). The 'stress luekogram' (Ives & Dacks 1956) is greatly reduced with Refinement of capture. For example, chimpanzees trained using positive reinforcement techniques, to cooperate with injection of anaesthetic to facilitate routine health assessment, did not display elevated circulating leukocyte profiles compared to captured animals (Lambeth *et al* 2006).

Other blood measures

Prolonged restraint has also been found to affect clinical chemistry measures circulating in blood. For example, cynomolgus macaques in two studies (Kissinger & Landi 1989; Landi *et al* 1990) showed elevated asparate and alanine aminotransferase (AST; ALT) in response to different restraints, with AST values rising sooner than ALT values. These two enzymes are commonly monitored during toxicological studies, it's therefore important to be able to distinguish between experimental effects, as a result of restraint, from drug-related changes indicative of hepatotoxicity, a common indicator of toxicity with many drugs during testing (Hall 2007).

c) Physical health

Physical health is an important requisite for maximising welfare (Webster *et al* 2004) and good scientific output. Poor health may sometimes be accompanied by negative subjective feelings such as pain, discomfort and distress (Hughes & Curtis 1997; Gregory 1998; Carstens & Moberg 2000; Chapter 2). Physical health is more than the absence of disease, it is a positive state of soundness of body, and it lies on a continuum from health through to subclinical disease, overt pathology, to a more serious change resulting in morbidity and mortality (Hughes & Curtis 1997). As with other species, cynomolgus macaques may develop a number of spontaneous disease conditions requiring diagnosis and veterinary intervention. It is not my aim here to review all possible indictors of illness, body damage or injury in cynomolgus macaques (see FELASA Working Party Report 1999; Lerche 2005; Lewis & Colgin 2005; Philipp & Purcell 2005; Wolfensohn & Honess 2005 for review),

but more general indicators of well-being that may be interpreted alongside behavioural and physiological indicators to give a more integrated account of welfare.

Macaque health should be monitored regularly by well-trained experienced care staff who are familiar with what's normal for that particular animal (Wolfensohn & Honess 2005). The process of monitoring health includes observations in the home pen and more obtrusive clinical examinations of the animals' appearance, general behaviour and demeanour. The CASE sponsor already has comprehensive health assessments in place for macaques. Where possible a formal quantitative assessment of animal health is preferred over qualitative methods - both methods are redundant, however, if the results of such assessments are not regularly reviewed and any problems dealt with swiftly. In the next sections I shall discuss quantitative assessments of the animals' appearance, fluctuations in body weight, and their relevance and usefulness as part of a comprehensive assessment of welfare.

Body weight

Body weight in laboratory housed cynomolgus macaques varies between colonies (e.g. juvenile males: 2027.9g ±339.4; females: 1935.4g ±272.2, Terao 2005; males: 2362.7g ±313.95; females: 2025.0g ±217.01, Andrade *et al* 2004) and males generally weigh more than females. Chronic pain, stress or disease can result in anorexia and/or associated weight loss or reduced weight gain (Bennett *et al* 1995; Wolfensohn & Honess 2005; Abee *et al* 2012). Percentage weight loss is considered an important indicator for establishing humane endpoints in studies (Morton 2000). For example ≥10% loss was used as an endpoint in parasitology studies conducted in baboons (Farah *et al* 2001), whilst OECD guidelines (2000) are less conservative for chemical testing, suggesting ≥20% loss over a few days or gradual persistent weight loss, being indicative of reduced food and or fluid intake, in response to animal suffering. In reality, losses of 10% are considered cause for concern and animals are more closely monitored and/or given supportive treatment where possible, rather than waiting for weight to drop to the 20% threshold. Close monitoring of the animal's body condition is also recommended (OECD 2000), as animals may lose body fat and muscle in chronic conditions but maintain a stable body weight.

Stress is well known to produce changes in body weight in animals (McLaren *et al* 2004). Experimental manipulations in nonhuman primates exposed to social stress consistently observe weight loss. For example, cynomolgus macaques previously housed alone for 10 weeks when placed into a new social group with unfamiliar individuals, rapidly dropped body weight; 6.1% over 3 days this was accompanied by a 40 bpm increase in heart rate compared to pre-mixing baseline levels (Strawn *et al* 1991). Previous studies using the same paradigm of social stress found reduced negative feedback in regulation of cortisol secretion resulting in hyper stimulation (Kaplan *et al* 2009; Shively *et al* 2009; Shively & Willard 2012), indicating the role of HPA in body weight loss.

As illustrated above, during stress, to meet the energetic costs of mounting a response, an individual uses stored reserves or diverts resources away from other bodily functions, resulting in weight loss, slowed growth, poor reproductive performance etc. (Moberg 2000; Shively & Willard 2012). Therefore the welfare implications of a given stressor will differ between animals depending on the level of stored reserves and their current pattern of energy expenditure (McLaren *et al* 2004). The costs of mounting a stress response in animals that are growing, a process which places additional energetic demands on them, may have increased welfare implications (McLaren *et al* 2004). Given that energy reserves are usually stored as fat, stress would cause body weight loss as energy stores are utilised (Moberg 2000). These effects are evident during air transport of macaques for research. Lee *et al* (2005a) found elevated levels of cortisol and blood leukogram in newly arrived sub-adult cynomolgus macaques. Furthermore, animals experienced significant weight loss (-11.7%) during the first week after arrival, with pre-transport body weights not recorded until week 3 of acclimatisation to the new facility.

Conversely, we might expect if we reduce stress experienced, an increase in body weight may be observed. However, the results of environmental enrichment, which is often employed to improve welfare (Newberry 1995; Young 2003; Buchanan-Smith 2010), does not always result in better weight gain or lead to increased body weight. For example, Schapiro and Kessel (1993), found a variable effect; with body weight gain observed in one enriched group and not another. Moreover, Bayne and colleagues (1991 b) found older animals gained weight with additional food enrichment

and provision of grooming boards. Conversely, physical enrichment was found to have no effect on body weight gain in nursery-reared rhesus monkeys (Clarke *et al* 1989). The type and presentation of enrichment may be critical to its success in ameliorating the effects of the laboratory environment and account for the differences in reported weight changes (Schapiro & Kessel 1993). Nonetheless, body weight change is potentially a useful measure of stress, and it would appear that body condition is a useful adjunct.

Body condition

Body condition is assessed by palpation and visual judgement as to the amount of muscle and subcutaneous fat (Clingerman & Summers 2005; Wolfensohn & Honess 2005). It can be used as guide to assess level of nutrition (Clingerman & Summers 2005), whether the animal is undergoing adequate growth, or experiencing poor health (e.g. chronic diarrhoea; Scarlett & Donoghue 1998; Clingerman & Summers 2005), and as described above it may be a useful parallel measure with body weight. Two published scoring methods for laboratory housed primates were developed in adult rhesus macaques (e.g. Clingerman & Summers 2005; Wolfensohn & Honess 2005). The interpretation of significance of body condition scores should be in light of the animal's current age and reproductive status. For example juvenile animals tend to be lean and lanky, scoring as underweight or thin. Moreover, an animal that has just undergone a growth spurt may be evaluated as thin whereas one whose growth has stabilized may be lean, as muscle mass catches up with previous bone growth (Clingerman & Summers 2005). The score system however, needs to be sensitive to pick up changes in growth and body weight with events and conditions in laboratories if it is to be a useful addition to welfare assessment (e.g. Study 2).

Hair loss (alopecia)

Hair loss can be common amongst captive primates, but is not so prevalent in wild animals (Honess *et al* 2005 a; Zhang 2011), suggesting that some aspect of the captive environment contributes to abnormal patterns of loss (Beisner & Isbell 2009). It has a complex aetiology and is a multifactorial problem with many contributing factors (reviewed in Beisner & Isbell 2009; Novak & Meyer 2009). Poor health, such as parasitic infections and skin diseases (Steinmetz *et al* 2005), nutrient specific deficient diets (Isbell 1995), reproductive conditions (Davis & Suomi 2006), hair pulling or over-

grooming (Reinhardt *et al* 1986; Reinhardt 2005) and social stress (Steinmetz *et al* 2006) have all been attributed as reasons for hair loss. It has been hypothesized that alopecia is the result of over-grooming or stereotypical trichotillomania due to boredom and/or stress (Steinmetz *et al* 2006). Treatment via various behavioural methods (e.g. additional environmental enrichment; Novak & Meyer 2009) does not always succeed in producing a reversal in severity (Kramer *et al* 2010), and therefore the link between hair loss and reduced welfare may be an oversimplification. Nonetheless, given its prevalence in captive animals, particularly in environments (social and physical) considered to be challenging to welfare and that recipients of hair pulling often show fear or avoidance reactions (Reinhardt *et al* 1986) which are likely to reflect poor welfare (Honess *et al* 2005 a), quantifying alopecia may be a useful tool for welfare assessment. Scoring involves estimating the severity and extent of hair loss according to a five point scoring system (Honess *et al* 2005 a).

3.1.2 Aims of the Chapter

Assessment of animal welfare is a critical component of Refinement. Given that it is multidimensional in nature, multiple measures are needed to give a more holistic assessment. Despite the large amounts of biological data generated from cynomolgus macaques used for regulator toxicology they are very rarely examined with animal welfare in mind. Furthermore, what is known about species-specific indicators is spread throughout the literature and not easily accessible to care staff. A first aim of the Chapter was to collate current knowledge on welfare assessment from the literature and to identify likely measures that can be incorporated into an assessment for cynomolgus macaques used in toxicology. A second aim was to integrate these measures and examine them in accordance to a framework (e.g. sensitivity, reliability, repeatability) to ascertain their fitness for purpose. My last aim was to identify behavioural measures that could be easily used by care staff to assess macaque welfare.

3.2 Methods

3.2.1 Animals, housing and husbandry

Animals

Juvenile and young adult (4.2.5a) purpose-bred male and female cynomolgus macaques of Mauritian origin from the same breeder were observed from arrival to start of study during the acclimatisation period and baseline recording of core battery measures (see animal demographics by study Table 3.2.1). Only stock animals were included in this study (e.g. naïve, un-dosed animals). Three studies were undertaken in a stepwise manner (Table 3.2.1; Section 3.2.3).

Table 3.2.1 Animal demographics for each study in the welfare assessment

Study	Cohort	No. of macaques		Age (w) (SD)	Acclim (w)	Tube	Brief description of study
		Male	Female				
1	1	16	16	132.5 (10.92)	9	3	Repeated measures • Comparing behavioural responses in home cage before & after handling & restraint for weighing & clinical observations, in weeks 4 & 7. • Linking behaviour during handling to changes in behaviour in the home pen after handling.
2	2 (A;B) Compared	A: 16 B: 8	A: 16 B: 8	A: 127.5 (5.83) B: 128.3 (6.70)	A: 10 B: 10+	3	Longitudinal • Sensitivity of APS & BCS to husbandry events. • Intra-observer reliability of APS & BCS. • Fluctuations in body weight during acclimatisation and pretreatment procedures. • Linking heart rate & blood pressure with behaviour during recording, changes in body weight, BCS & APS.
3	2 (A;B) Pooled	A:32 B:16	A:32 B:16	A: 123.9 (11.90) B: 124.3 (9.00)	A: 10 B: 10	A: 3 B: 5	Single event • Linking heart rate, haematology, clinical chemistry parameters, & body weight, body condition, alopecia score change with behaviour during restraint.

Age: mean age (SD) on arrival; **Acclim:** length of acclimaisation time; **Tube:** Number of tube habituation sessions; **W:** Week: Mean (SD); **BCS**: Body condition score; **APS:** Alopecia score; Tables 3.2.2 - 9; Section 3.2.

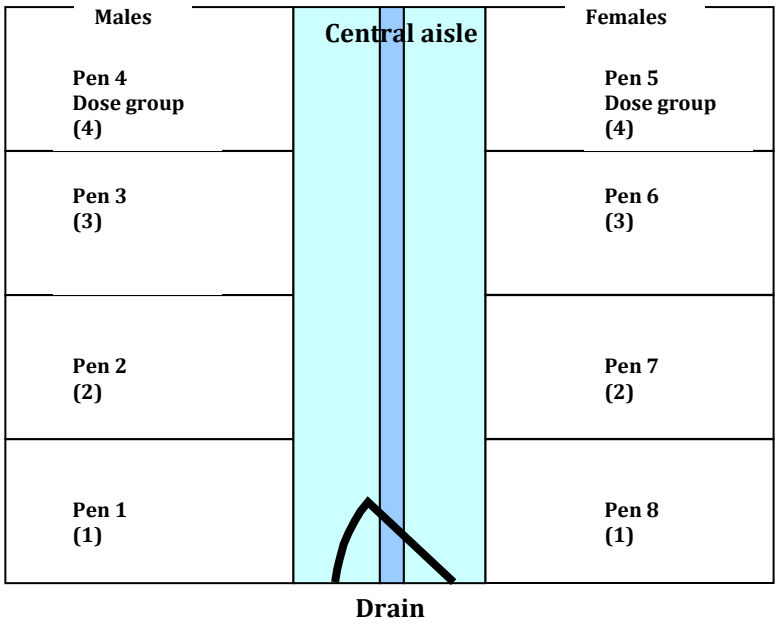
Housing

Macaques were housed in single rooms and cared for in accordance with the Animals (Scientific Procedures) Act (1986), associated codes of practice (Home Office 1986) and in-house standard operating procedures. Animal rooms were maintained at temperature range: 18-24°C, relative humidity: 30-80%, air ventilation: minimum 10 air changes per hour. Lighting was by fluorescent tubes on automated 12 hour light/dark (L/D) cycle: 06.00-18.00h (L). Monkeys were kept in single-sex groups from arrival, and in large enriched pens (Figures 3.2.1 a - c): eight per room arranged four each side of a central aisle containing a drain. Pens measured 2.25m^2 floor area x 2.4m high, with solid floors, sides and backs. Floors were covered with sawdust (depth approximately 1.5 cm). Pens had opaque Perspex partitions 0.75m from the wall to the front of the pen through which

macaques had visual contact with neighbouring pens. Three suspended slatted wooden platforms (0.75m^2 floor area) running side to side at 0.5 and 1.5m high on the back of the pen and running back to front at 1.0m high on the left of the pen, along with a solid wooden veranda (0.1m^2) above the pen door provided perching opportunities. Weld mesh covered the roof of the pen and cage fronts were solid to 0.3m high, with metal bars running vertically in the walk-in door, and horizontally on the left hand side. Transport boxes could be mounted on to a hatch located at 1.3m on the left hand side. This provided ease of capture and transportation.

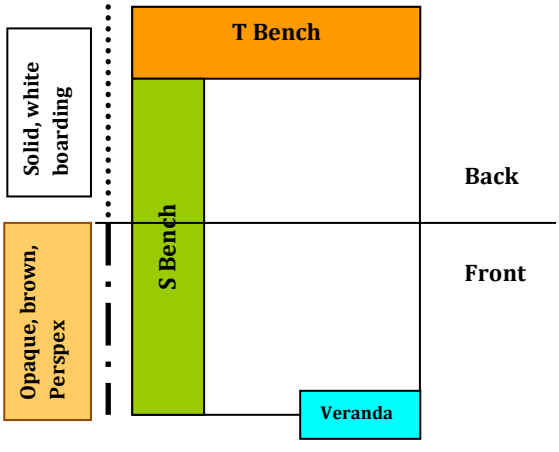
Figures 3.2.1 a - c Schematic views of home room and pen layout. ***Not to scale.***

a) Home room layout.



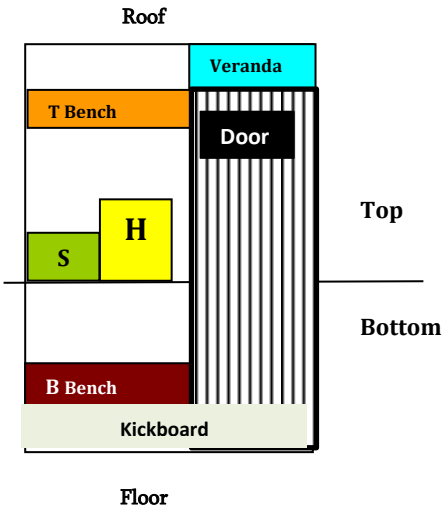
Eight pens per home room, males (M) on the left, females (F) on the right, pens contain macaques assigned to ascending dose groups (1-4). Central aisle is approximately 1 m wide with a covered drain running down the middle, parallel to the pens.

b) Pen layout. ***View from above.***



T(op) Bench: Wooden slatted perch located on the back wall of the pen.
S(ide) Bench: Wooden slatted perch located on left of the pen, runs the length of the pen.
Veranda: Solid wooden perch located on the right above the door.
Back: Back half of the pen corresponds with the edge of the solid white boarding that makes up the wall of the pen.
Front: Front half of the pen corresponds with the edge of the opaque brown Perspex that makes up the wall of the pen.

c) Pen layout. ***View from the front.***



T(op) Bench; S(ide) Bench; Veranda : as (b).
Hatch (H): in the front bars of the pen. Allows animals to run into a transport box.
Top (T): Top half of the pen, corresponds with the lower edge of the side bench to the roof of the pen.
Bottom (B): Bottom half of the pen, corresponds with the lower edge of the side bench to the floor of the pen.
Floor: Floor of the pen.
Roof: Roof of the pen.

Husbandry

A detailed husbandry schedule is given in Chapter 5 with discussion on quantity of macaque-staff contact. In brief, macaques were fed daily a standard pelleted commercial primate diet (approximately 100g/monkey; SQC Mazuir Primate Diet, Special Diet Services, Witham) supplemented with a range of food stuffs; 25g bonio biscuit (Spillers) per animal, fresh fruit and vegetables, forage mix, peanuts, raisons, sunflower seeds (fresh and dried supplements were fed on alternative days). Food was presented at intervals throughout the day and scattered in the pen. Macaques were given additional plastic dog and primate toys, rotated each day for enrichment. Fresh water was provided daily in two water bottles mounted at 0.4 and 1.3m on the front of the cage, available *ad libitum* and supplemented daily with diluted blackcurrant fruit drink. Macaques were visually inspected by care staff at intervals throughout the day (Chapter 5).

Central aisles were scraped and hosed daily and home pens were powerwashed weekly. On powerwashing days macaques were caught into transport boxes (Chapter 5) and placed in the play room. The play room was identical to home room in terms of pen layout and dimensions with additional suspended toys and bamboo-like perches provided along with plastic dog and primate toys placed into water-filled plastic paddling pools. The home room was thoroughly cleaned and bedding replaced. Animals were returned to their home room (caught and transported in transport boxes) after approximately 2h in the playroom, and after weighing and physical examination by technical staff (Table 3.2.2).

3.2.2 General methods for individual parameters in the welfare assessment

Table 3.2.2 gives a summary of recording methods included in the welfare assessment. Sections a-h contain supplementary information.

Table 3.2.2 Overview of sampling and recording techniques for welfare assessment.

Welfare variable	Recording & analysis method
Body condition score (BCS) ***Section a***	Scored by PhD student (0-5; with half points in between; Table 3.2.3), visual inspection and manual palpation of amount of subcutaneous fat and muscle mass covering bony prominences (e.g. thorax, lumbar and thoracic areas of spine, hips and pelvis)
Alopecia score (APS) ***Section b***	Scored by PhD student (1-5; Table 3.2.4), visual assessment of pelage and pattern of hair loss on the animals back.
Clinical observation (CO) ***Section c***	Animals physically examined on arrival and weekly throughout the acclimatisation period. Clinical code (details abnormality) and location manually recorded by technician on exam.
Body weight (BW) ***Section d***	Animals were weighed on arrival and weekly throughout acclimatisation period and before dose on day 1 of study (Figure 1.3). Recorded electronically from Sarturius Combics 3 digital scales (CWP39).
Heart rate (HR) ***Section e***	Recorded by the ECG technician. At least 30 seconds of digital trace were recorded from awake macaques using Ponemah 4.4 physiological platform (Data Service International, Valley View, Ohio) and amplifier (ACQ-16 Parallel Legacy). A six lead ECG recording was made and heart rate was derived from Lead II (Appendix 1.3). **Restraint** – Tube (Chapter 5). **HR calculation -** Mean heart rates (bpm) derived from 7 R-R peaks (6 heart beats; Appendix 1.3) were calculated by the ECG analyst.
Blood pressure (BP) ***Section e***	Recorded by the ECG technician and veterinarian. Five consecutive blood pressure recordings were taken from awake macaques, immediately after ECG recording, using Memo diagnostic (MD_15/90 Pro) high definition oscillometry (HDO) blood pressure monitor (S+B medVet GmBH, Babenhauesn, Germany; Appendix 1.5). **Restraint** – Tube. **BP calculation** - Systolic (SYS), Diastolic (DIA) and mean arterial pressure (MAP) were recorded by ECG technician at time of acquisition.
Haematology & clinical chemistry (H&CC) ***Section e***	Performed by technician. Single-use disposable hypodermic fixed needle attached to disposable plastic syringe, sized according to total volume of blood withdrawn (study dependent). **Venepuncture site** – Femoral vein or artery. **Restraint** – Tube. **Analysis** – Conducted by the CASE sponsor. Automatic and manual blood smear (Table 4.2.5, method C).
Behavioural Observations (BO) ***Sections f – g: response to handling & restraint*** ***Section h: in home-pen recording***	Recorded by the PhD student. Observations were conducted of macaque behaviour during handling and restraint and in the home cage for matched time points pre and post handling before, during and after clinical observations and weighing. Sampling methods and a summary of behaviours recorded are outlined in Sections f-h and Tables 3.2.5 – 9 c. Behaviours were recorded by direct observation during handling and restraint and remotely for in-home cage observations using four, 6mm lens, high resolution, external colour infrared day/night CCTV cameras (Voltek: VPCDN2), with 10m infrared resolution and automatic switch to black/white for night-time recording to maintain good visual acuity of subjects. Images were recorded onto a standalone four channel digital video recorder with 160 GB harddrive and played back on 24" flatscreen colour monitor (Samsung).

(i) Physical health

a) Body condition score (BCS)

Body condition scores were awarded according to the methods outlined in Clingerman and Summers (2005), and Wolfensohn and Honess (2005 a) (Table 3.2.3). This was to allow comparison between the two, to determine which of the methods were most sensitive to body weight change in juvenile and young adult macaques. Both published methods were developed in adult rhesus macaques.

b) Alopecia scores (APS)

Alopecia was scored according to the methods outlined in Honess *et al* (2005 a). The animal's hair was gently stroked upwards and downwards once, visually inspected and scored for missing or thinning hair (Table 3.2.4).

Body condition and alopecia scores were awarded for each animal, once it had been removed from the transport box, before technicians conducted clinical observations, to avoid bias.

c) Clinical observations (CO)

The technical staff worked systematically from head to tail visually and physically inspecting the animal for signs of abnormality, illness or injury. Clinical observation codes were used to describe deviations from normal, recorded onto an automated data capture system. Animals were then weighed.

d) Body weight change (BW)

Animals were placed individually into a weigh box on an electronic weigh scale linked up to a PC. Their body weight was recorded automatically onto an in-house data capture system. Animals were then removed from the box and returned by hand to their home pen.

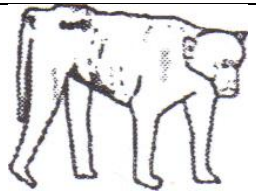
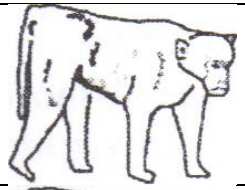
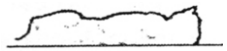
Body condition, alopecia scores, clinical observations and body weights were transferred into a Microsoft excel (2007) spreadsheet for data screening.

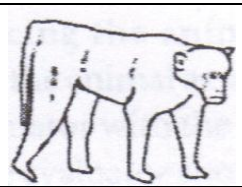
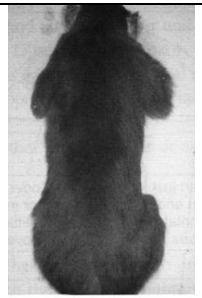
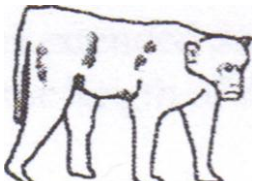
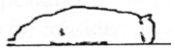
(ii) Physiological parameters

e) Baseline measures in the core battery

Heart rate, blood pressure (Chapter 5; Appendix 1.1 - 1.5), haematology and clinical chemistry analytes (Chapter 4) were recorded by methods detailed elsewhere in the thesis. All parameters were recorded by CASE sponsor staff onto Microsoft excel (2007) spreadsheets.

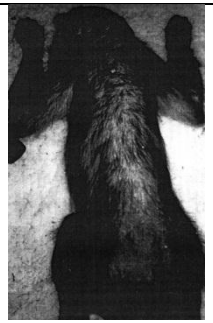
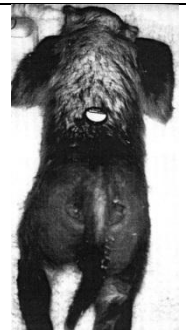
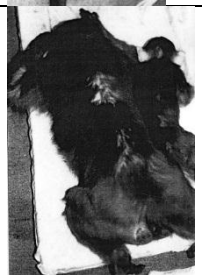
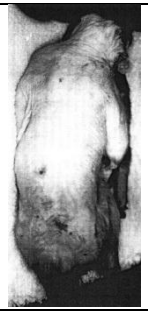
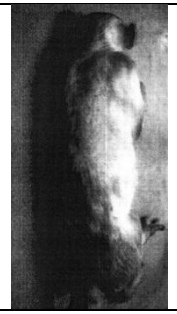
Table 3.2.3 Comparison of published body condition scores (Clingerman & Summers 2005; Wolfensohn & Honess 2005).

Score	Clingerman & Summers (2005)			Wolfensohn & Honess (2005)		
	Description	Ambulating view	Right, back lateral view	Score	Description	Back view
1 Emaciated	Hips, facial bones, spinous processes & ribs very prominent. Minimal-no muscle mass over ilium or ischium. Anus recessed between ischial callosities. Body very angular, no subcutaneous fat.			0 Emaciated	No description	
1.5 Very thin	Hips, spinous processes & ribs prominent. Very little muscle mass over hips & back. Anus may be recessed between ischial callosities. Body angular, no subcutaneous fat.			1 Severely underweight	Vertebrae sharp & prominent, distinct gap between each.	
2 Thin	Hips, ribs & spinous prcesses easily palpable. Small muscle mass over hips and lumbar region. Minimal subcutaneous fat.			2 Underweight	Vertebrae smooth & prominent, gap detectable between each.	
2.5 Lean	Hips & spine feel firm. Hips & spinous processes readily palpable, not prominent. Body less angular. Thin layer of subcutaneous fat.					

3 Optimum	Hips, ribs & spinous processes palpable, not visible. Well-developed muscle mass & subcutaneous fat layer. No abdominal, axillary or inguinal fat pads.			**3 Normal**	Vertebrae smooth & round, & slightly prominent.	
3.5 Slightly overweight	Hip bones and spinous processes palpable with firm pressure but are not visible. Bony prominences smooth. Rib contours are smooth and only palpable with firm pressure. Small abdominal fat pad may be present.					
4 Heavy	Hips, spinous processes & difficult to palpate. Bony contours smooth, less defined. Fat deposits accumulating in axillary, inguinal or abdominal areas.			**4 Overweight**	Vertebrae just detectable with pressure.	
4.5 Obese	Hips & spinous processes difficult to palpate. Bony contours smooth, poorly defined. Prominent fat pads inguinal, axillary or abdominal region. Abdomen pendulous.					
5 Grossly overweight	Hips, ribs & spinous processes palpable with deep pressure. Large fat pads abdominal, inguinal and axillary regions. Pronounced fat deposits.			**5 Obese**	Vertebrae not detectable.	

Clingerman & Summers 2005: *Macaca mulatta*; Scores awarded based upon palpation & visual inspection of whole animal; Wolfensohn & Honess 2005: *M. mulatta*: Scores awarded based upon palpation of lumbar spine only.

Table 3.2.4 Method for alopecia scoring macaques (*M. mulatta*; Honess *et al* 2005).

Score	Description	Back view		Score	Description	Back view	
1	Good coat condition, complete cover.			**4**	Generalised alopecia > 50% of back		
2	Small patches of alopecia (2-5cm^2).			**5**	Back completely bald, more skin visible than hair.		
3	Large patches of alopecia (≥ 5 cm^2), or numerous small ones totalling 25 – 50% of the back						

Alopecia scores based on severity and proportion of visible hair loss on the back.

(iii) Behavioural observations

f) Handling and restraint (for weighing and clinical observation by technician)

Observation protocol

All data collection took place when animals were being handled for weighing and clinical observation at weekly intervals. I stood in the treatment room approximately 1.5m away from the macaques and technicians handling them; this gave me a good view of the macaques' behaviour without causing further alarm during handling. I was dressed in overalls to match technical staff. At any one time there were 2 - 3 technicians present in the room, one handing animals, one recording animal health and body weight data, and one technician preparing to remove the next animal from the transport box.

The series of events were as follows: animals were captured as a group (n = 3 – 5) from the same pen into a single transport box, placed onto trolley and wheeled from the playroom to the treatment room. Each animal was then gently removed in-turn by a single technician and transferred to a weigh box placed on electronic scales; body weight data were captured automatically. Upon removal from the weigh box the animal was visually inspected from head to toe by the technician and any physical departures from normal recorded by entering a prescribed code into the data capture system. Animals were then taken by hand, by the technician, back to the home pen. It took approximately 30 seconds for the individual monkey to be removed from the transport box, weighed and examined.

Data collection

In order to avoid interference with sensitive electrical equipment in the CASE sponsor's treatment room (particularly during ECG and blood pressure recordings), all handling and restraint observations were recorded onto a check sheet. Check sheets were designed according to the recommendations made by Martin and Bateson (2007). A handheld digital stopwatch was used for determining time intervals for time sampling. Behaviour was recorded according to the sampling method outlined in Table 3.2.5.

Sampling methods

By necessity, focal animal sampling was used to record the behaviour of macaques during handling, using the mutually exclusive behavioural categories presented in Table 3.2.8. The behavioural categories were defined following a short pilot study. The recording rule used was instantaneous time sampling for macaque facial expression with all occurrences (Martin & Bateson 2007) of the remaining behavioural categories (vocalisations, displacement activities and elimination behaviour), whereby each occurrence of the behaviours are recorded during the observation session. This approach was used to give true frequencies of occurrences of behaviours of short duration or those likely to occur only rarely.

The sampling interval was kept fairly short (10 seconds) to further reduce the probability that short duration behaviours would be missed. It was also a matter of practicality, the total recording session is short (30s) as the handling procedure was rapid, but continuous recording was somewhat impractical too as the view of the animal was obscured by technical staff performing their duties, and it would necessitate the observer to be stood next to the handling technician, which was unsafe and unfeasible for all parties.

At each sample point a subjective category of active or passive (Table 3.2.8) that describes the animals response to handling ('handleability') was awarded by the observer.

Table 3.2.5 Time sample points for recording behaviour during handling for weighing and clinical examination by technician.

Sample points (code)	Sample interval (s)	Description
T1	-	First sample point. Facial expression recorded upon exit from the transport box, once animal fully out of the box (legs clear of the box) and held by one or both arms by the technician. Scored handleability.
T2	10	T1 +10s Facial expression recorded, scored handleability
T3	10	T1 +20s
T4	10	T1 +30s

Data processing

For each animal, macaque facial expressions recorded using instantaneous time sampling method, were reported as a proportion of all sample points (e.g. X/4) on which the behaviour occurred, and the frequency (count) of all occurrences behaviour (e.g. vocalisation, displacement, elimination) were summed per 30 second observation window.

(g) Tube restraint (for recording ECG, blood pressure, and venepuncture)

Observation protocol

As for (f) all data collection took place whilst animals were being placed into and restrained in a tube for recording ECG and HDO blood pressure followed by venepuncture in Study 3 only. Observations were recorded in a similar manner to (f), and the series of events was as follows: animals were captured as a group from the same home pen into a single transport box, placed onto trolley and wheeled to the treatment room. Each animal was then gently removed in turn by a single technician and transferred into a restraint tube (held by another technician), and placed into a cradle on the table. ECG electrodes were attached according to the method outlined in Appendix 1.3. Animals were gently supported and prevented from exiting the tube by a technician holding their legs. Once ECG data had been recorded electrodes were removed and a cuff placed around the macaques tail. Blood pressure was recorded according to Appendix 1.5. Once completed, the monkey was returned by technician to the home pen.

Data collection & sampling methods

As (f).

Table 3.2.6 Time sample points for recording behaviour during handling and restraint for recording ECG and blood pressure parameters.

Sample points (code)	Sample interval (s)	Description
T1	-	First sample point. Facial expression recorded upon exit from the transport box, once animal fully out of the box (legs clear of the box) and held by one or both arms by the technician. Scored handleability.
T2	Variable.	Second sample point. Facial expression recorded as soon as the animal is restrained manually to enter tube (Chapter 5). Scored handleability.
T3	Variable.	Third sample point. Facial expression recorded as ECG electrodes placed on the skin. Scored handleability
T4	10	T3 +10 Facial expression recorded during ECG. Scored handleability.
T5	10	T3 + 20
T6*	10	T4 + 30
T7	Variable.	Seventh sample point. Facial expression recorded as blood pressure cuff is placed on macaque's tail (Appendix 1.5). Scored handleability.
T8	Variable.	Eighth sample point. Facial expression recorded as cuff begins to inflate (Appendix 1.5). Scored handleability.
T9	10	T8 +10 Facial expression recorded during HDO. Scored handleability.
T10	10	T8 + 20
T11	10	T9 + 30
T12	10	T10 + 40

Sample points T8 – T12 coincide with acquisition of systolic, diastolic and mean arterial blood pressures (Appendix 1.5) * see text.

For Study 3 only, animals did not have blood pressure recorded, but venepuncture immediately followed removal of ECG electrodes. Following on from sample point T6 above *behavioural

responses were recorded according to the sample points outlined in Table 3.2.7 for the remainder of the procedure.

Table 3.2.7 Time sample points for recording behaviour during handling and restraint for venepuncture.

Sample points (code)	Sample interval (s)	Description
T7*	Variable.	First sample point. Facial expression recorded as needle pierces the skin. Scored handleability.
T8	10	T7 + 10s T7 + 20s T7 + 30s Facial expression recorded during venepuncture. Scored handleability
T9	10	
T10	10.	

Data processing

As (f).

Table 3.2.8 Behavioural categories and definitions used for cynomolgus macaques recorded during restraint and handling for weighing and clinical observations, ECG and blood pressure recording, and during venepuncture.

	Categories	Elements	Description
Facial expression	Fearful	Fear-grin	Mouth open – although varies in degrees. Awarded a rating from 1 – 3: Fear**3** – Mouth fully open; both upper and lower teeth exposed, lips retracted; Fear**2** – Partial open mouth; Upper and lower teeth partially exposed, lips partially retracted; Fear**1** – Just open mouth; Only bottom set of teeth partially exposed; Eyes may be fixed or flick to and from handlers, repetitive "peeking".
	Submissive	Lip-smacking	Clearly audible lip-smacking noise accompanies movement of jaw, lips and tongue; Eye brows pulled back; The head may or may not be tilted up or bobbed repeatedly up and down.
	Aggressive	Aggressive	Raised eyebrows, fixed look; Slightly open mouth, teeth not usually displayed; Tense lips, with corners of the mouth brought forward, so that the mouth forms a circular aperture; Ears are flattened.
	Alert	Alert facial	Subtly different to the relaxed face; Fully open eye, eyes may flicker to and from handlers, muscle tone and skin tension around the mouth. Mouth is closed.
	Relaxed	Relaxed facial	All facial elements (eyes, lips etc.) are in a "neutral" position. No visible skin or muscle tone evident to the observer. Mouth is closed.
Calls	Vocalise		Animal emits any kind of vocalization audible to the observer. Animal must also be seen to vocalize. Vocal sounds separated between 2 seconds will be scored as a separate vocalization.
		Kra	3 – 4 sound pulses, rasping like quality to the sound.
		Kra Alarm	Modification of "KRA" – 3 – 5 sound pulses, harsh and quickly repeated.
		Wraagh	Harsh call: tonal followed by harsh components.
		Scream	Loud vocal discharges, variable acoustic structure.
		Khreet Screech	Loud Screech noise, tonal scream, longer sounding than a scream, often repeated.
		Squeal	High-pitched.
		Whimper	Analogous to the pant threat in rhesus macaques; HA-HA-HA sounding noise with a quiet but raspy quality.
		Coo	Relatively quiet calls includes: High-long-calls; low-long-calls; high-short calls; low-short-calls; High-extended-calls – variation on OOOH sound.
Elim	Elimination	Urination	Void urine during handling or restraint.
		Defecation	Void faeces during handling or restraint**.** Score faecal consistency: 0 – normal; 1 – loose; 2 - watery.
Handling	Bite handler	Contact or intentioned	Animal bites the handlers' glove, or makes intentioned movements even if contact is not possible, bite is directed towards handler. Each bite is scored as the animal closes its mouth.
	Overall reaction: *handleability*		Subjective score awarded by observer.
		Active	Animal actively tries to resist handler during handling or restraint: struggles, wriggles, tries to escape from handler or restraint position.
		Passive	Opposite of above: animal does not actively resist handling or restraint.
	Duration of restraint		Total time (seconds) animal restrained for procedure (time starts as soon as animal is held in restraint position until released from the position)
Other	Other		Any behaviour not listed in the above descriptions. Describe context and occurrence.
	Comments		Comments on external events perceived by the observer as affecting animal's behaviour.

Description of macaque facial expressions adapted from van Hooff (1967) & Redican (1975) and vocalisations from Palombit (1992a, b).

h) Pre- and post- handling (for weighing and clinical observation by technician)

Observation protocol

To determine whether exposure to a known laboratory stressor (e.g. handling and restraint) would produce measurable behavioural changes in macaques, matched behavioural data were recorded at the same time points on the day before and the day after handling. All data collection took place in relation to animals being handled for weighing and clinical observation. This was to avoid the confounds of test article and its administration, as following venepuncture or ECG/BP recording animals may be dosed at the onset of regulatory studies. It was not possible therefore to get pre- and post-handling responses in relation to venepuncture and ECG/BP. Moreover, to determine whether macaques were acclimatising to conditions in the laboratory, the observations were repeated for the same animal on week 4 and week 7 after arrival. This was to avoid TB testing in week 3 and to observe animals the week before ECG and BP recording.

Weighing and clinical observation for stock animals usually occurred in the afternoon (approximately 1300h) when all procedural work had been completed. Macaques were weighed out of the playroom, being returned to the home room after it had been cleaned and powerwashed (Section 3.2.1). Behavioural data were recorded for 2h post stressor, and before technicians gave final feed and performed last checks (approx. 16.30). In addition, behaviours were observed overnight (after lights out and before lights on: 18.00-06.00h; see Crockett *et al* 1995). Animals were marked ventrally (stomach and chest) with a coloured spray vegetable dye, livestock marker (Ritchey Ltd., N. Yorkshire) to identify individuals in the group. This enabled behaviour of macaques during handling to be matched to their response pre- and post-handling. Overnight, black and white images were recorded remotely (Table 3.2.2) as this gave the best visual acuity in recorded images. It was impossible therefore to identify individuals, so behavioural responses were recorded at group level.

Data collection

It was difficult to record behaviour directly in the home room. The narrow central aisle and pen layout meant macaques spent a lot of time attending to me. Moreover, a single observation window in the door to the room only provided views of pens 1 and 8. Therefore remote recording of

macaque behaviour in the home pen was chosen to avoid confounds of my presence on the animals' behaviour. This was particularly likely with newly acquired animals that were still habituating to care staff and the new laboratory environment. I chose to record behaviour remotely using CCTV cameras mounted on the weld mesh roof of the room to the left or right of centre of the drain, outside and midway between pens 1-2, 3-4, 5-6, 7-8. The camera field of view gave sufficient coverage of 2 pens (provided they were mounted as far back as possible but remaining out of reach of macaques in opposite pens). Table 3.2.2 details camera and recording equipment. Behaviours were recorded from played back images, on to THE OBSERVER (v. XT; Noldus, The Netherlands) installed on a laptop which made for faster data entry and quicker analysis. Behaviours were recorded according to the sampling method outlined in Table 3.2.9 a.

Table 3.2.9 a Summary table of behavioural recording methods in response to handling and restraint for weighing and clinical observations in weeks 4 and 7.

Recording event	**Description, schedule and duration of recording**					**Behaviours recorded**	**Recording method**
	Day 1	***Day 2***					
	Camera On: 11.30 -camera Off: 09.00 (day2).	10 am macaques moved to playroom.	Capture, handling restraint. Duration for room approx. 40 min.	20 minutes post last animal returned. Start 2h remote recording from pens.	Night-time recording from pens. Start: 18.30; Finish: 05.30h.		
	Recording schedule						
Pre - (matched to post; day 2)	√Retrospectively matched to post (day 2). 2h observation window.	X	X	X	Retrospectively matched to post (day 2).	Table 3.2.9 b	2h pre - observation sessions. Remote. Focal animal. Instantaneous time sampling and all occurrences. Night-time behaviours: Focal group. Scan sampling. Instantaneous time sampling.
Handling & restraint	X	X	√ Duration: 30s per animal.	X	X	Table 3.2.5	Direct. Focal animal. Instantaneous time sampling and all occurrences
Post - (day 2)	X	X	X	√ Duration 2h.	√	Table 3.2.9 b	2h post - observation sessions. As pre -.

Sampling methods

Focal animal sampling was used to match the behaviour of macaques during handling and restraint with pre- and post- restraint comparisons in the home pen. The mutually exclusive behavioural categories are presented in Table 3.2.9 b. Owing to the reduced visual acuity in the night-time recordings, individual animals could not be identified and so a smaller range of behaviours were recorded (following Crockett *et al* 1995; Table 3.2.9 c). The recording rule used was instantaneous time sampling at 15-second intervals, which was used for recording all behaviours apart from abnormal and aggressive responses, for which all occurrences were recorded. In addition animal location and whether its gaze was directed to the door of the room were recorded at each sample interval. Data collection sessions lasted 5 minutes and were scheduled every 30 minutes for 2 hours post-handling and restraint, beginning 20 minutes after the last animal was returned to the room. This was to avoid any further disturbances by technicians completing checks post-handling. The order of focal animal sampling followed the order with which the animals were handled and returned to the home pen; the first animal returned was the first to be observed etc. Pre-handling behaviour observations were matched in time and order to the post-handling observations retrospectively.

Similarly for the group observation at night-time, scan samples were recorded every 15s for each 5-minute recording session, every 30 minutes, from 30 minutes after lights out (18.30h) until 30 minutes before lights on (05.30h); chosen to avoid activity associated with settling down for the night or waking up in the morning in anticipation of lights-on (unreported observations from pilot studies).

Data processing

As (f), for each animal, behaviours recorded using instantaneous time sampling were reported as a proportion of all sample points on which the behaviour occurred. Behaviours were collapsed into 1-hour cohorts after handling, and to before and after midnight for night-time recordings (Crockett *et al* 1995). All occurrences of behaviour were summed per collapsed observation window.

Data analysis

Data for all three studies were screened for normality and transformed if needed, to perform parametric statistics (see Section 4.2.6i for more details). Non parametric statistics were used if transformations were unsuccessful.

Table 3.2.9 b Behavioural categories recorded during home pen observations, 2h pre- and post-handling and restraint for weighing and clinical observations.

Categories	Elements	Description
Instantaneous time sampling every 15 seconds		
Activity	Inactive alert	Animal is stationary; lying, sitting and awake. Animal is not in contact (within 1 arms' reach) with another individual.
	Inactive relaxed	Animal is stationary; lying, sitting, awake or sleeping, eyes may be open or closed, but not scanning or following other individuals. Animal is not in contact (within 1 arms reach) with another individual.
	Huddle	Animal is stationary and huddled to another individual: 1 or more surface is in physical contact with another individual.
	Embrace	Animal is stationary and in an embrace with another individual. Two individuals clasp each other face-to-face contact between ventral surfaces (chest or head) arms of one or both individuals involved are wrapped around the body of the embrace partner. This may be unilateral or mutual.
	Contact	Animal is stationary and awake, and in contact with another individual – within 1 arms reach of another animal.
	Agitated locomotion	Animal moving rapidly between locations, with a stiff un-relaxed gait and tense musculature.
	Relaxed locomotion	Animal moves between locations by walking, climbing, running or swinging, relaxed gait when moving and observe relaxed muscle tone.
	Play	Animal engages in high activity interaction (chase, rough and tumble) with individuals involving non-aggressive physical contact.
	Aggression - cage mates	Animal engages cages in bodily contact or facial threat directed towards another individual. May include chase, slaps, hair pulling, open-mouthed display, direct stare.
	Aggression - cage	Any vigorous shaking or banging of the cage with feet (most common) or hands.
	Explore	Animal investigates other non-food related items (using fingers or mouth) – these may be toys, pen furniture (e.g. benches) and the sides of the pen.
	Auto-groom	Fingers (hands), lips or teeth are drawn through the individuals own coat, skin or teeth.
	Allo-groom	Grooming other animals: the hands and or lips are drawn through the coat, skin or teeth of another individuals coat, skin or teeth.
Appetitive behaviours	Feeding	Feeding, foraging, looking for food.
	Drinking	At the drinker nozzle
Elimination	Urination	Void urine.
	Defecation	Void faeces.
All occurrences during 5 minute collection session		
Displacement	Self-scratching	Individual's hand or foot scratch part of his or her own body. Scratching was scored as a new event after 2 second interval with no scratching.
	Body shake	Dog-like body shake. Whole body involved.
Abnormal	Stereotypic	Repeated, unvarying behaviours. Include circling, pacing, bar-strumming, pen rubbing.
	Abnormal	Include self-directed behaviours: Hair plucking, self-mutilation or injurious behaviour (e.g. tail biting, biting limbs, removing fingernails, chewing fingers) directed towards self.
Other	Other	Any behaviour not listed in the above descriptions. Describe form and context.
	Comments	Comments regarding events in the environment or problems with recording.
Recorded at 15 second intervals		
Vigilance	Watch door	Animal visually attending to the door of the room whilst stationary or moving. Can be engaged in other activities.
Location (Figures 3.2.1 b & c)	Veranda	Animal on the veranda.
	Upper	Animal is in the upper half of the pen.
	Lower	Animal is in the lower half of the pen.
	Floor	Animal on the floor of the pen.
	Front	Animal is in the front half of the pen.
	Back	Animal is in the back half of the pen.

Table 3.2.9 c. Behavioural categories recorded during night-time, in home-pen observations.

Category	Description
Instantaneous time sampling every 15s	
Active	Animal moves between locations by walking, climbing, running or swinging.
Asleep	Sitting with head down, lying down.
Inactive awake	Sitting, perching, including head up, looking around.
Eat	Picking up, holding food.
Drink	At the drinker nozzle.
Other	Not included above.
Recorded at 15 second intervals	
Location Veranda **Upper** **Lower** **Floor** **Front** **Back**	Animal can be engaged in any other activity Animal on the veranda. Animal in the upper half of the pen. Animal in the lower half of the pen. Animal on the floor. Animal is in the front half of the pen near to the observer. Animal is in the back half of the pen away from the observer.
Social contact **Contact** **Alone**	Animal can be engaged in any other activity Animal within one arms reach of another includes animals that are huddling and embracing. Animal not within one arm's reach of another.

After Crockett *et al* (1995).

3.2.3 Examining the sensitivity, reliability and repeatability of welfare measures

I undertook three studies in a step wise manner to systematically examine the sensitivity, reliability and repeatability of individual behavioural, physiological and physical health parameters to provide an overall assessment of welfare in the cynomolgus macaque.

3.2.3a Study (1) Macaque behavioural responses to handling and restraint for husbandry procedures during acclimatisation

i) Study aims

1. To identify behavioural changes in response to a common laboratory stressor, i.e. handling and restraint for weighing and clinical observation.

2. To observe, with acclimatisation to the laboratory environment, whether the behavioural responses to handling and restraint change over time.

3. To examine the relationship between behavioural responses and changes in body weight recorded weekly from macaques.

4. To examine relationships between behavioural responses in week 7 with fluctuations in body weight, heart rate, blood pressure, and behavioural responses during handling and restraint for ECG and HDO recording procedures.

ii) Method

Animals

A single cohort of animals of Mauritian origin macaques from the same breeder that entered the unit at the same time were included in this study (Table 3.2.Study 1). They were recorded on weeks 4, 7 and 8 during pretreatment procedures.

Table 3.2.Study 1 Animal demographics for study (1)

Cohort	N (M:F)	n/pen	A (w)		BW (Kg)		Acclim (w)	Welfare assessment data collected
			M	F	M	F		
A	32 (16:16)	5/3/3/5 males & females*	135	132	2.5	2.3	9	BWC; HR; BP; BO handling (ECG & BP), BO pre-post handling

N: Total number of animals included in the study; **M:** Male; **F:** Female; **n/pen:** Number of macaques per pen; *macaques were grouped for study requiring treatment free animals in groups 1 & 4. **Age:** Age of animal (weeks) on arrival; **BW:** Mean body weight on arrival (Kg); **Acclim:** Length of acclimatisation time (weeks): time from arrival to day 1 of dose; **Welfare assessment data:** Welfare related data gathered from each cohort for comparison; **BWC:** Body weight change (g; weekly); **HR:** Heart rate (bpm); **BP:** Blood pressure (mm/Hg); **BO handling:** Behavioural observations of handling and restraint for recording ECG and HDO; **BO** pre- post - handling: Behavioural observations pre and post handling in the home pen.

Recording methods and schedule

Macaque body weights and clinical observations were recorded weekly by technicians from arrival during acclimatisation and on day 1 of study before dose (week 9; Table 3.2.2). I recorded behavioural responses during (1) handling and restraint for weight and clinical observations on weeks 4 and 7 after the animals arrived in the unit, and (2) pre- and post- handling observations for those occasions, and (3) during recording for ECG and HDO data collections (week 8). Behaviours were recorded as outlined in Sections 3.2.2; Tables 3.2.2; 5; 6; 8; 9 a, b, c. Electrocardiograms and HDO blood pressures were recorded by methods outlined in Table 3.2.5.

Data analysis

Independent t-tests (unequal variance assumed for between-Cohort analysis) were used to compare differences in age and body weight on arrival between male and female macaques. One-way repeated measures Analysis of Variance (ANOVA) with sex as a between-subject factor and Bonferroni *post hoc* tests were performed for each behaviour recording from week 4 and 7 to track changes in behaviour with acclimatisation time on the unit.

There were too few animals (N = 32) to perform a multiple linear regression (Section 4.2.9) and systematically examine the effects in changes of multiple measures of welfare (e.g. combination of behaviour during recording, change in body weight, week 7 behavioural responses) on heart rate and blood pressure. Instead, pairs of welfare variables were plotted against one another and correlated (Pearson's or Kendalls' tau) to determine their significance. One-way ANOVAs were performed on mean heart rates, blood pressures and behavioural responses during handling and restraint to determine if there were any sex-related differences.

3.2.3b Study (2) Determining the sensitivity and reliability of body condition and alopecia during acclimatisation

i) Study aims

1. To track changes in body condition, alopecia and body weight during acclimatisation in relation to husbandry and pretreatment procedures.
2. To determine the intra-observer reliability of two body condition and one alopecia scoring method(s).
3. To determine which body condition scoring method is most sensitive, and appropriate for use in juvenile and young adult macaques.
4. To examine the relationship between fluctuations in body weight, body condition and alopecia, with heart rate and blood pressure, and behavioural responses during handling and restraint for ECG and HDO recording procedures.

ii) Method

Animals

Two cohorts (A & B) of Mauritian origin macaques from the same breeder that entered the unit at the same time were included in this study (Table 3.2.Study 2a). They were followed from arrival during acclimatisation, and Cohort A was observed during pretreatment procedures prior to onset of study. The commercial study for Cohort B was delayed, hence pretreatment data were not examined, and although not a complete match to Cohort A, owing to fewer animals, they provide a useful comparison for identifying the effects of scientific procedures on body condition, alopecia

and body weight change. Cohorts A and B were housed in separate but adjacent rooms and experienced the same husbandry procedures albeit staggered, as they occurred on alternate days.

Table 3.2.Study 2a Animal demographics for study (2)

Cohort	N (M:F)	n /pen	A(w)		BW (Kg)		Acclim (w)	Welfare assessment data collected
			M	F	M	F		
A	32 (16:16)	4	130	125	3.26	3.07	10	BCS; APS; BWC; HR; BP; BO handling (ECG & BP).
B	16 (8:8)	4	132	125	3.32	3.04	10+	BCS; APS; BWC.

N: Total number of animals included in the study; **M:** Male; **F:** Female; **n/pen:** Number of macaques per pen; **Age:** Age of animal (weeks) on arrival; **BW:** Mean body weight on arrival (Kg); **Acclim:** Length of acclimatisation time (weeks): time from arrival to day 1 of dose; **Welfare assessment data:** Welfare related data gathered from each cohort for comparison; **BCS:** Body condition score; **APS:** Alopecia score; **BWC:** Body weight change (g; weekly); **HR:** Heart rate (bpm); **BP:** Blood pressure (mm/Hg); **BO handling:** Behavioural observations of handling and restraint for recording ECG and HDO.

Recording methods and schedule

Macaque body weight and clinical observations were recorded weekly by technicians from arrival, during acclimatisation and on day 1 of study before dose (Table 3.2.2). I recorded two body condition scores for comparison (e.g. Clingerman & Summers 2005 and Wolfensohn & Honess 2005) and recorded severity of alopecia, where present (e.g. Honess *et al* 2005) whilst macaques were weighed and examined (not including arrival; Section 3.2.3i; Tables 3.2.3; 3.2.4). The intra-observer reliability of body condition and alopecia scores were determined from repeated scoring of the same animals whilst they were sedated for tuberculin (TB) testing in week 3. Cohort A and B were TB tested at the same time and their data pooled. Sedated animals were taken by the technician to the treatment room for testing by the veterinarian before being returned to their home pen. As animals were transported to and from the treatment room, I recorded their tattoo, located on the inner thigh, and awarded two scores for body condition and one for alopecia. At least five technicians, working in rotation, were involved in transporting macaques to ensure speedy completion of the procedure before the macaques awoke. The speed, number of technicians and number of animals involved meant the order of presentation for both scoring opportunities were different. It was difficult to recognise tattoo identification. The confound of observer bias was further avoided by recording observation 1 and 2 in separate check sheets, covered by a blank sheet of paper. In total 48 macaques were scored twice, this exceeds the minimum number of animals required to assess intra-observer reliability with an expected reliability of 0.8 or higher at 0.05 significance (Walter *et al* 1998).

As macaques in Cohort A were assigned to study, the opportunity arose to examine the relationship between multiple welfare measures (e.g. change in body condition, alopecia, body weight with heart rate and blood pressure and behavioural responses during procedures), and cohort B provided a control group for comparison. Electrocardiogram (ECG) and high definition oscilometry (HDO) were used to record heart rate and blood pressure in macaques, as part of the core battery of baseline (pretreatment) measures taken before dosing (Table 3.2.2; Section 3.2.2).

Data analysis

Independent t-tests (unequal variance assumed for between-Cohort analysis) were used to determine differences in age and body weight on arrival between male and female macaques within and between Cohort A and B. To identify changes in body condition, alopecia and body weight with acclimatisation and pretreatment procedures, one-way repeated measures Analysis of Variance (ANOVA) with Bonferroni *post hoc* tests and sex as a between-subject factor were performed on each Cohort. Univariate analyses, run with sex as a covariate, were performed to compare between Cohorts weekly changes in body weight. To determine the intra-observer reliability for pairs of body condition and alopecia scores, the percentage of agreement and kappa coefficient (Cohen 1968) were calculated. Kappa provides a way describing by how much the scores differ. For example larger disagreements between scores are considered to be more important than those that are closer (but not perfectly) in agreement (Byrt *et al* 1993). A kappa coefficient <0.4 indicates poor agreement, whilst > 0.6 is considered good (Habbison *et al* 2002).

In order to examine the relationship between body condition and body weight Pearson's correlations were performed on scores derived from the different methods (e.g. Clingerman & Summers 2005; Wolfensohn & Honess 2005). This, along with tracking changes in body condition and body weight over the acclimatisation and pretreatment period allowed determination of the sensitivity of each method when scoring juvenile and young adult macaques.

As with Study 1, there were too few animals (N = 32) in Cohort A to perform a multiple linear regression (Section 4.2.9) and examine the effects in changes of multiple measures of welfare. Pairs of welfare variables were correlated (Pearson's) to determine their significance, and One-way

ANOVAs were performed on mean heart rates, blood pressures and behavioural responses to determine sex-related differences.

3.2.3c Study (3) Linking multiple measures of welfare during pretreatment procedures

i) Study aims

1. To examine the relationship between behavioural responses during restraint with heart rate, haematological and clinical chemistry parameters.

ii) Method

Animals

Two cohorts (A & B) of Mauritian origin macaques from the same breeder that entered the unit at the same time were included in this study (Table 3.2.Study 3a). They were only observed during pretreatment procedures; ECG recording and venepuncture to obtain samples for analysis of haematological and clinical chemistry parameters, as part of baseline core battery measures (Table 3.2.2). Animals were housed in neighbouring rooms (Table 3.2.Study 3a), and were assigned to two different studies. Pretreatment procedures were performed over 3 days, a room at a time.

Table 3.2.Study 3a Animal demographics for Study (3).

Cohort	N (M:F)	n/pen	Age (w) M	Age (w) F	Acclim (w)	Welfare assessment data collected
A	32 (16:16)	4	123.7	123.9	10	HR, H&CC, BWC, BO during data collection.
B*	64 (32:32)	4	124.4	124.6	10	

* Macaques were housed in two separate rooms as described in Section 3.2.1. **N:** Total number of animals included in the study; **M:** Male; **F:** Female; **n/pen:** Number of macaques per pen; **Age:** Age of animal (weeks) on arrival; **Acclim:** Length of acclimatisation time (weeks): time from arrival to day 1 of dose; **Welfare assessment data:** Welfare related data gathered from each cohort for comparison; **BWC:** Body weight change (g; weekly; 2w pretreatment period only); **HR:** Heart rate (bpm); **H&CC:** Haematology and clinical chemistry; **BO handling:** Behavioural observations of handling and restraint for recording ECG and venepuncture.

Recording methods and schedule

Animals were restrained in a clear Perspex tube. Blood was withdrawn from the femoral artery or vein after removal of ECG electrodes, before animals were released from the tubes, weighed and returned to their home pen. Percentage body weight change was calculated from body weight data recorded by technicians in weeks 8 - 10 (e.g. two week pretreatment period). Electrocardiogram and venepuncture were performed on day of weighing in week 9. Animals were habituated to the

tube restraint on consecutive days preceding ECG. This involved placing the animals in the tube and on to a cradle on a table for 1 minute, before returning them to their home pen. Animals in Cohort A were habituated across three sessions and animals in Cohort B were habituated for five. The differences in habituation sessions were due to another study examining the efficacy of increasing the number of prior experiences of tube restraint on cardiovascular data (Chapter 5).

Data analysis

The effects of habituation were not examined directly in this study; however, along with the potential effects of other (predictor) variables on heart rate, haematology and clinical chemistry parameters a multivariate regression model (Section 4.2.6; 4.2.9) was used to examine the relationship between behavioural responses and baseline measures in the core battery (Table 3.2.Study 3b). Kendall's tau (non-parametric) correlations were performed to identify statistically significant relationships between behaviour during restraint and individual haematological, clinical chemistry and heart rate variables.

Table 3.2.Study 3b Defining known predictor variables and outcome variables for multiple regression.

Outcome (biological) variable				Predictor variables			
Variable	**Unit**	**Description**	**Regression model**	**Variable**	**Unit**	**Description**	**Regression model**
Haematology				Cohort	-	Categorical	A (3)
Hb	g/dL				-		B (4)
RBC	mil/cmm			Sex		Categorical	Male (1)
PCV	%						Female (2)
PLAT	1000/cmm			Age	W	Continuous	Entered as
WBC	10^9/L						continuous
N	10^9/L						data
L	10^9/L			Habituation	W	Continuous	
Clinical			Entered as				
chemistry		Continuous	continuous				
AST	IU/L		data	Fear 3			
ALT	IU/L			Fear 2			
ALP	IU/L			Fear 1			Entered as
GgT	IU/L			Alert	-	Continuous	continuous
UREA	mmol/L			Relax			data
TBILI	mmol/L			Active			
CREAT	mmol/L			Passive			
TPROT	g/L			Kra			
GLOB	g/L			Wra			
				Scr			
HR	bpm			Whi			
BWC	%						

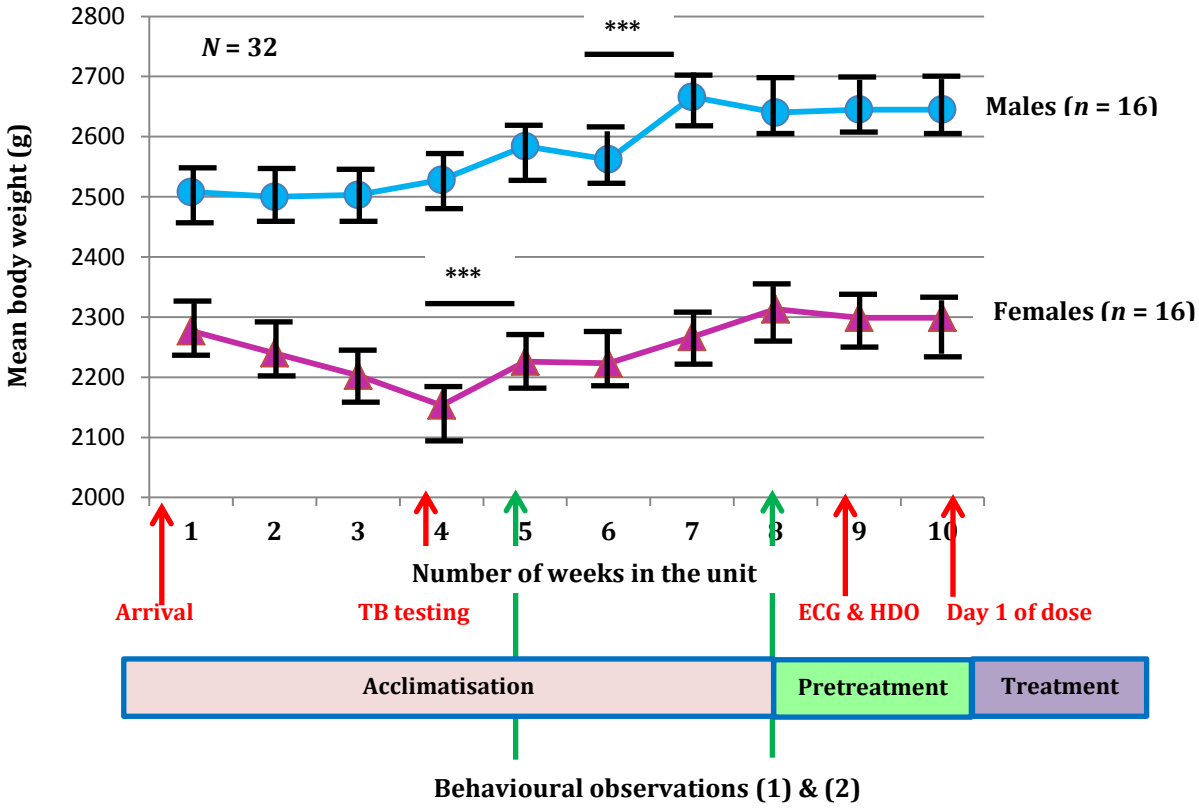
W: weeks. Vocalisations: Kra, Wra, Scr, Whi showed colinearity with Fear3 and were excluded from the model.

3.3 Results

3.3.1 Study (1)

Animals were generally in good health. Incidences of loose faeces were recorded by technicians in five females during weeks 1 – 3; they quickly resolved without any treatment. Moreover, two males and five females were recorded as having small amounts of missing fur on their backs or top of their head on arrival. The mild severity remained the same throughout acclimatisation, and hair loss was thought to be associated with a skin blemish that occurred before entry on the unit. Males and females were the same age (Table 3.2.Study 1), but males were heavier on arrival and throughout acclimatisation (males: 2508.13g ±132.48; females: 2276.88g ±114.99, $t(30) = 5.27$, $p<0.001$), and the pattern of change varied significantly (Figure 3.3.1 a). Males were heavier by week 10 than when they arrived ($f(1) = 12.17$, $p<0.05$), whereas females showed a non-significant weight gain.

Figure 3.3.1 a Fluctuations in body weights of males and females during acclimatisation and pretreatment period.



Males did not start to gain weight after arrival until week 3 on the unit, whilst females gradually lost weight until week 4. This was found to be related to weight loss in animals with loose faeces. Females then showed a period of 'catch up growth', gaining significant weight in week 4 - 5 (3.4% ± 6.24; $f(1) = 94.37$, $p<0.001$. Males gained most weight between weeks 6 – 7 (4.1% ± 6.76; $f(1) =$

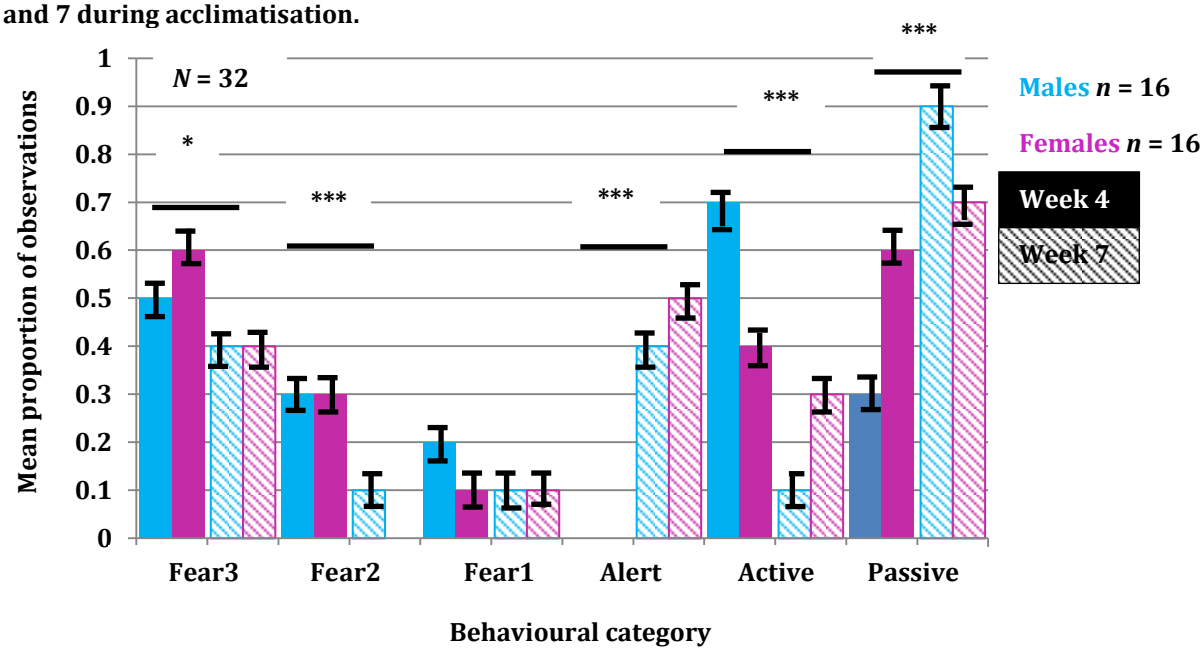
38.23, $p<0.001$). Body weights of males and females levelled out during the pretreatment period and onset of ECG and HDO recording.

Behavioural responses

(a) During handling

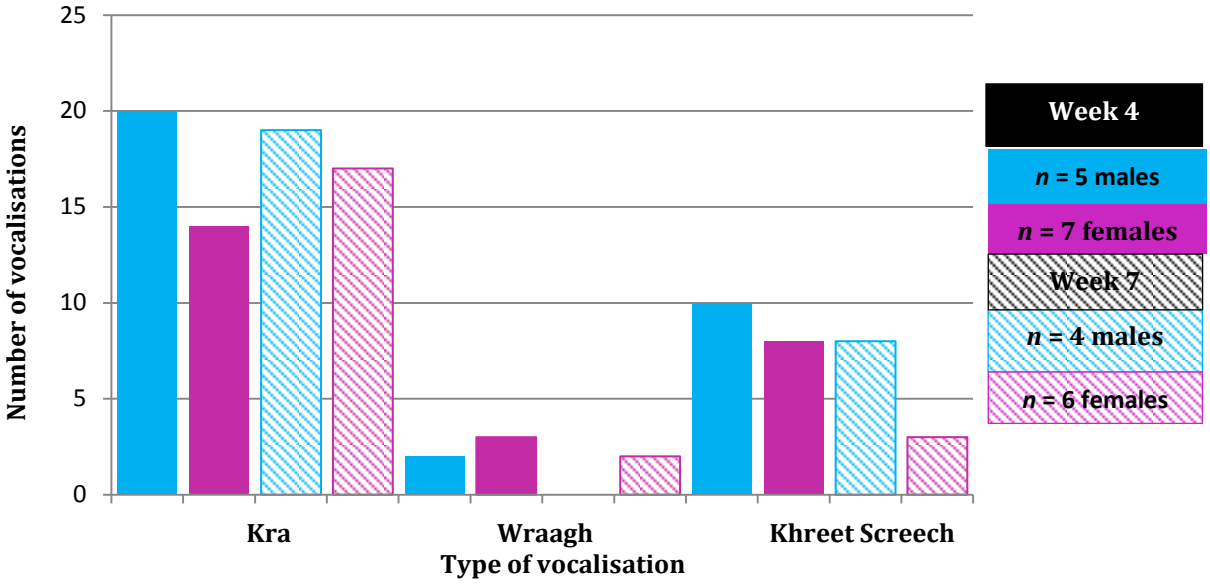
The behavioural responses of macaques during handling for weighing and clinical observation changed significantly over time. Behaviour of males and females were not found to differ significantly (Figures 3.3.1 b).

Figure 3.3.1 b Mean behavioural responses of male and female macaques recorded in weeks 4 and 7 during acclimatisation.



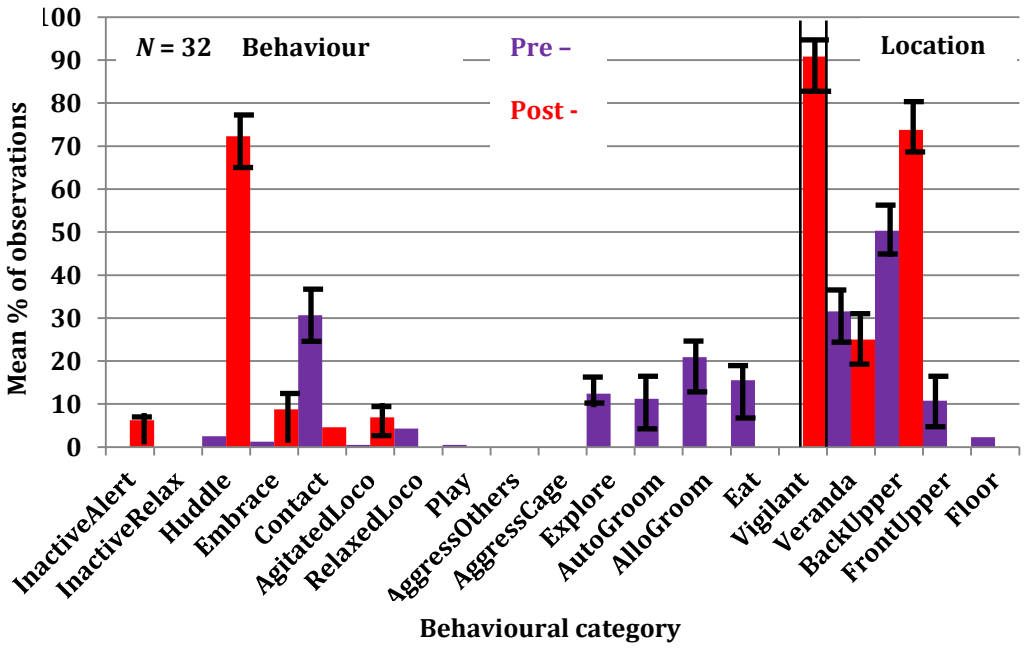
Fear3 ($f(1) = 3.78$, $p<0.05$), Fear2 ($f(1) = 20.68$, $p<0.001$) and Alert ($f(1) = 36.02$, $p<0.001$) facial expressions, along with active ($f(1) = 52.39$, $p<0.001$) and passive ($f(1) = 19.84$, $p<0.001$), responses during restraint altered significantly with time spent in the unit. Macaques became more passive and less fearful. Furthermore ten animals ($n = 4$ males; $n = 6$ females) vocalised during handling in week 7, compared to 13 animals ($n = 5$ males; $n = 7$ females) in week 4 (Figure 3.3.1 c). Vocalisations were given during removal from the transport box, and with Fear3 facial expression. Kra, Wraagh and Khreet Screech vocalisations were given by the same animals each week. Percentage body weight change was found to be moderately, positively correlated with Wraagh vocalisations given during handling in week 4 ($r^2 = 0.30$, $p<0.05$).

Figure 3.3.1 c Number of vocalisations given by macaques during handing in weeks 4 and 7.



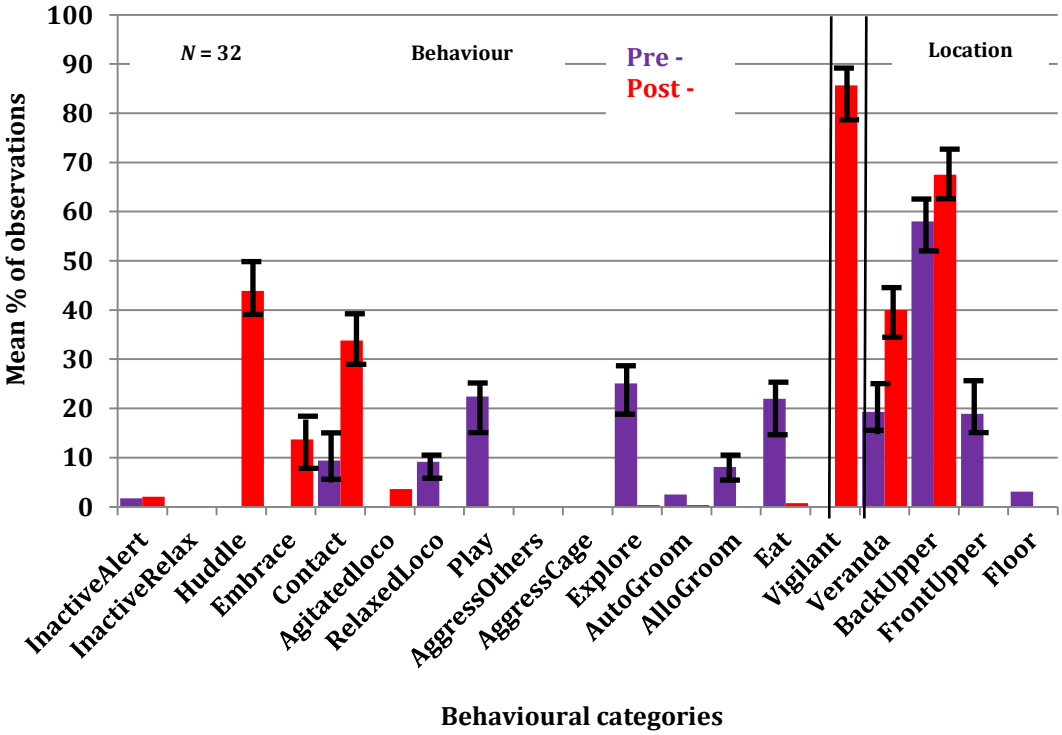
(b) Pre- and post- handling responses

Figure 3.3.1 d Mean behavioural response of male and female macaques recorded during pre – and post – handling during Week 4 acclimatisation.



There were no significant differences between hour 1 and hour 2 in pre - handling observations. Inactive alert ($f(1) = 21.99$, $p<0.001$) and vigilant ($f(1) = 5.45$, $p<0.005$) were significantly higher in hour 1 compared to hour 2 during post handling observations. All but three behaviours (embrace, agitated locomotion and play) and two locations in the pen (veranda and back upper) were found to significantly ($p<0.05$) differ between pre – and post – handling observations in the home pen. After handling macaques spent more time huddling, vigilant at the back of the pen. They also had a less varied behavioural repertoire compared to pre-handling observations.

Figure 3.3.1 e Mean behavioural response of male and female macaques recorded pre – and post – handling week 7 acclimatisation.



There were no significant differences between hour 1 and hour 2 in pre - handling observations. Inactive alert ($f(1) = 4.16$, $p<0.05$), embrace ($f(1) = 12.72$, $p<0.05$), explore ($f(1) = 8.40$, $p<0.05$) and eating ($f(1) = 8.40$, $p<0.05$) differed between hour 1 and hour 2 during post handing observations. During hour 1 after handling macaques were more inactive alert and embraced more than in hour 2, whereas they explored and spent more time eating in hour 2 than hour 1 after handling. The behaviour of macaques differed significantly ($p<0.05$) after handling compared to pre - handling baseline observations in all but inactive alert behaviours. In common with week 4 observations, macaque behaviour during post – handling was less varied when compared to matched time points pre – handling. After handling macaques spent more time huddling, in contact, being vigilant at the back of the pen, or on the veranda.

The differences between weeks 4 and 7 acclimatisation, in pre – and post – handling observations recorded from animals in their home pen are displayed in Table 3.3.1 a.

Table 3.3.1 a Comparison between pre – and post – handling observations in weeks 4 & 7.

Behaviour	Percentage of observations (%)			
	Pre -		Post -	
	Week 4	Week 7	Week 4	Week 7
Inactive alert	0	1.7	6.3	3.0
Inactive relaxed	0	0	0	0
Huddle	2.5	0	72.3 $f(1) = 5.9, p<0.05$	34.9
Embrace	1.3	0	8.8	22.8 $f(1) = 6.25, p<0.05$
Contact	30.7 $f(1) = 8.43, p<0.05$	9.4	4.6	35.2 $f(1) = 8.73, p<0.05$
Agitated locomotion	0.5	0	6.9	2.1
Relaxed locomotion	4.3	9.1 $f(1) = 6.15, p<0.05$	0	0
Play	0.5	22.4 $f(1) = 18.40, p<0.05$	0	0
Explore	12.4	25.1 $f(1) = 16.69, p<0.05$	0	0
Auto groom	11.3 $f(1) = 6.69, p<0.05$	2.5	0	0.6
Allo groom	20.9 $f(1) = 5.96, p<0.05$	8.1	0	0
Feeding	15.5	22.0	0	0
Vigilance	0	0	90.9	95.6
Location				
Veranda	31.5	19.3	25.0	35.4
Upper back	50.3	58.0	73.8	70.8
Upper front	10.8	18.9	0	0
Floor	2.3	3.1	0	0

By week 7 acclimatisation macaques spent less time in contact, more time in relaxed locomotion, playing and exploring than matched time points pre – handling in week 4. They also spent less time auto and allo-grooming in week 7 compared to week 4. Post - handling, macaques spent less time huddling and more time embracing and in contact in week 7 observations compared to week 4.

Night-time behaviours

The difference between weeks 4 and 7 behavioural responses recorded at night before midnight and from midnight onwards in pre – and post – handling conditions are shown in Tables 3.3.1 b - d.

Table 3.3.1 b Night-time pre - and post - handling behavioural observations in weeks 4 & 7.

Week	Observation	Before midnight (18.30 – 23.30h)			Midnight onwards (00.00 – 05.30h)		
		Percentage of scan samples (%)			Percentage of scan samples (%)		
		Asleep	Awake	Active	Asleep	Awake	Active
4	Pre -	81.5	18.3	0.0	66.0	14.8	19.2
	Post -	75.0	10.0	15.0	50.0	35.8	13.7
7	Pre -	91.7	8.3	0.0	76.1	14.7	9.1
	Post -	82.7	17.3	0.0	60.0	15.5	24.5

Observation: Pre - /post – behaviour observations in relation to handling & physical examination by technician (Table 3.2.9 a); **Asleep, Awake, Active**: Defined in Table 3.2.9 c. Male & female data pooled.

Table 3.3.1 c Pairwise comparisons before midnight.

Behaviour	W4		W7		Pre -		Post -	
	Pre -	Post -	Pre -	Post -	W4	W7	W4	W7
Asleep	$f(1) = 18.60$, $p<0.05$		$f(1) = 7.04$, $p<0.05$			$f(1) = 18.50$, $p<0.05$		$f(1) = 17.50$, $p<0.05$
Awake	$f(1) = 5.79$, $p<0.05$			$f(1) = 3.12$, $p<0.05$	$f(1) = 18.30$, $p<0.05$		$f(1) = 3.20$, $p<0.05$	
Active		$f(1) = 56.79$, $p<0.05$						$f(1) = 33.89$ $p<0.05$

Asleep, Awake, Active: Defined in Table 3.2.9 c. Male & female data pooled. **W4:** Week 4; **W7:** Week 7; **Pre - /Post –** behaviour observations in relation to handling & physical examination by technician (Table 3.2.9 a); denotes % scan sample observations were significantly higher.

Table 3.3.1 d Pairwise comparisons midnight onwards.

Behaviour	W4		W7		Pre -		Post -	
	Pre -	Post -	Pre -	Post -	W4	W7	W4	W7
Asleep	$f(1) = 16.64$, $p<0.05$		$f(1) = 74.84$, $p<0.05$			$f(1) = 12.30$, $p<0.05$		$f(1) = 13.18$, $p<0.05$
Awake							$f(1) = 12.36$, $p<0.05$	
Active	$f(1) = 8.61$, $p<0.05$			$f(1) = 8.50$, $p<0.05$		$f(1) = 15.12$, $p<0.05$		$f(1) = 18.91$, $p<0.05$

Asleep, Awake, Active: Defined in Table 3.2.9c. Male & female data pooled. **W4:** Week 4; **W7:** Week 7; **Pre - /Post –** behaviour observations in relation to handling & physical examination by technician (Table 3.2.9 a); denotes % scan sample observations were significantly higher.

Macaques spent less time asleep before and after midnight following handling in weeks 4 and 7. Before midnight, they spent more time active after handling in week 4, and more time awake in week 7. After midnight, macaques spent more time awake and active in weeks 4 and 7.

Significant correlations between behavioural response during handling and pre – post-handling observations

Multiple correlations were performed to see if there were any relationships between behaviours observed during handling and restraint and pre -/post – responses in the home pen. Significant correlations are displayed in Table 3.3.1 e.

Table 3.3.1 e Significant correlations between behavioural observations in weeks 4 & 7.

W	Correlation	r^2	Interpretation
4	% inactive alert post – handling is negatively correlated with Fear2 during handling.	-0.45, $p<0.05$	Greater proportion of Fear2 given during handling less time spent inactive alert after handling.
	% inactive alert post – handling positively correlates with Active during handling.	0.41, $p<0.05$	Greater proportion Active during handling the more time spent inactive alert after handling.
	% inactive alert post – handling negatively correlates with Passive during handling.	- 0.42, $p<0.05$	Greater proportion passive during handling the less time spent inactive alert after handling.
	% auto groom post – handling positively correlates with Khreet Screech given during handling.	0.53, $p<0.05$	Higher number of Khreet Screech vocalisations given during handling the greater time spent auto grooming after handling.
	% asleep post - <24.00h negatively correlates with Wraagh vocalisations during handling.	-0.42, $p<0.05$	More time spent asleep before midnight after handling the less Wraagh vocalisations heard during handling.
	% awake post - <24.00h positively correlates with Wraagh vocalisations during handling.	0.34, $p<0.05$	More time spent awake before midnight after handling the more Wraagh vocalisations heard during handling.
	% active post - <24.00h positively correlates with Wraagh vocalisations during handling.	0.40, $p<0.05$	More time spent active before midnight after handling the more Wraagh vocalisations heard during handling.
7	% agitated locomotion post - handling positively correlates proportion of Wraagh vocalisations given during handling.	0.48, P<0.05	The greater frequency of Wraagh vocalisations heard during handling the more time spent in agitated locomotion after handling.
	% relaxed locomotion post -handling negatively correlates with Fear1 facial expression given during handling.	-0.43, p<0.05	The greater proportion of Fear1 facial expression given during handling the less time spent performing relaxed locomotion after handling.
	% explore post - handling negatively correlates with Fear1 facial expression given during handling.	-0.48, P<0.05	The greater proportion of Fear1 facial expression given during handling the less time spent performing exploration after handling.
	% play post - handling negatively correlates with Fear1 facial expression given during handling.	- 0.48, P<0.05	The greater proportion of Fear1 facial expression given during handling the less time spent performing play after handling.
	% asleep pre - <24.00h negatively correlates with Wraagh vocalisation during handling.	- 0.36, $p<0.05$	More time spent asleep before midnight, on the night before weighing the less Wraagh vocalisations heard during handling for weighing and physical examination.

W: week number.

Physiological responses

Cardiovascular parameters recorded in Study 1 are given in Table 3.3.1 f; male and female, heart rates and blood pressures did not significantly differ.

Table 3.3.3 f Heart rate and blood pressure of macaques in Study (1).

Sex	N	Cardiovascular measure	Mean (±SD)	O-R
Male	16	**Heart rate (bpm)**	224.8 (24.9)	165-252
		Systolic (mm/Hg)	135.1 (12.2)	108-155
		Diastolic (mm/Hg)	76.6 (9.3)	63-92
		Mean arterial pressure (mm/Hg)	97.4 (9.2)	82-113
		Pulse (bpm)	238.1 (27.4)	175-284
Female	16	**Heart rate (bpm)**	219.9 (27.2)	159-253
		Systolic (mm/Hg)	144.1 (22.1)	119-197
		Diastolic (mm/Hg)	83.3 (13.3)	63-116
		Mean arterial pressure (mm/Hg)	105.5 (15.4)	85-144
		Pulse (bpm)	234.9 (25.9)	176-268
All	32	**Heart rate (bpm)**	222.3 (25.8)	159-253
		Systolic (mm/Hg)	139.6 (18.1)	108-197
		Diastolic (mm/Hg)	79.9 (11.8)	63-116
		Mean arterial pressure (mm/Hg)	101.5 (13.1)	82-144
		Pulse (bpm)	236.5 (26.6)	175-284

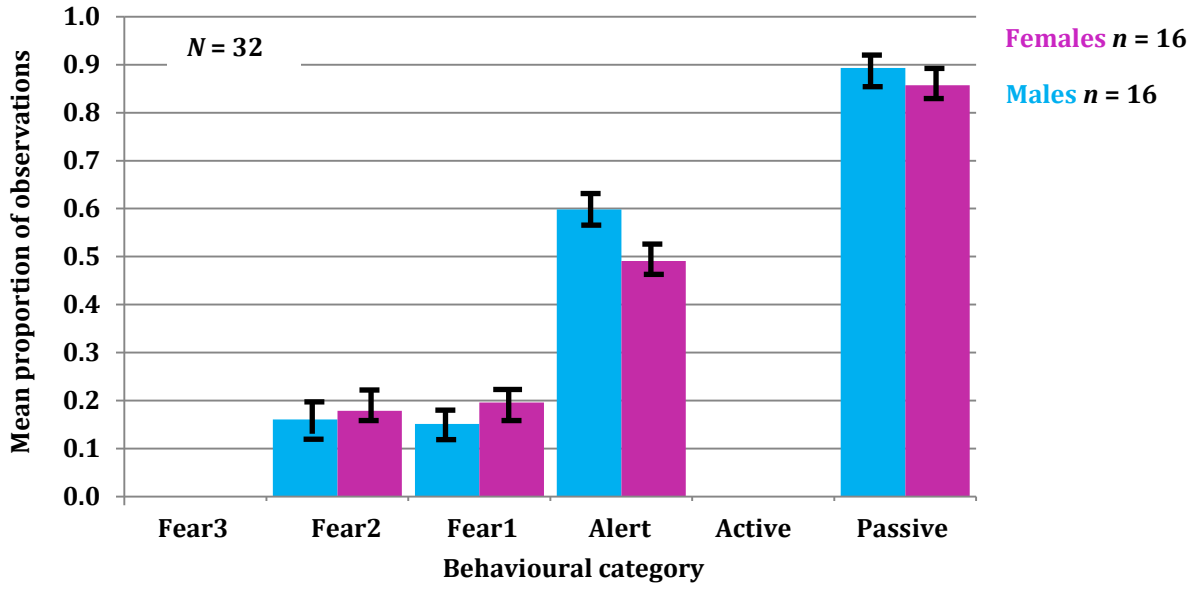
N: number of animals; **O-R:** observed range. Cardiovascular measures did not differ significantly (p>0.05) between males and females.

Behavioural responses during ECG and HDO recording

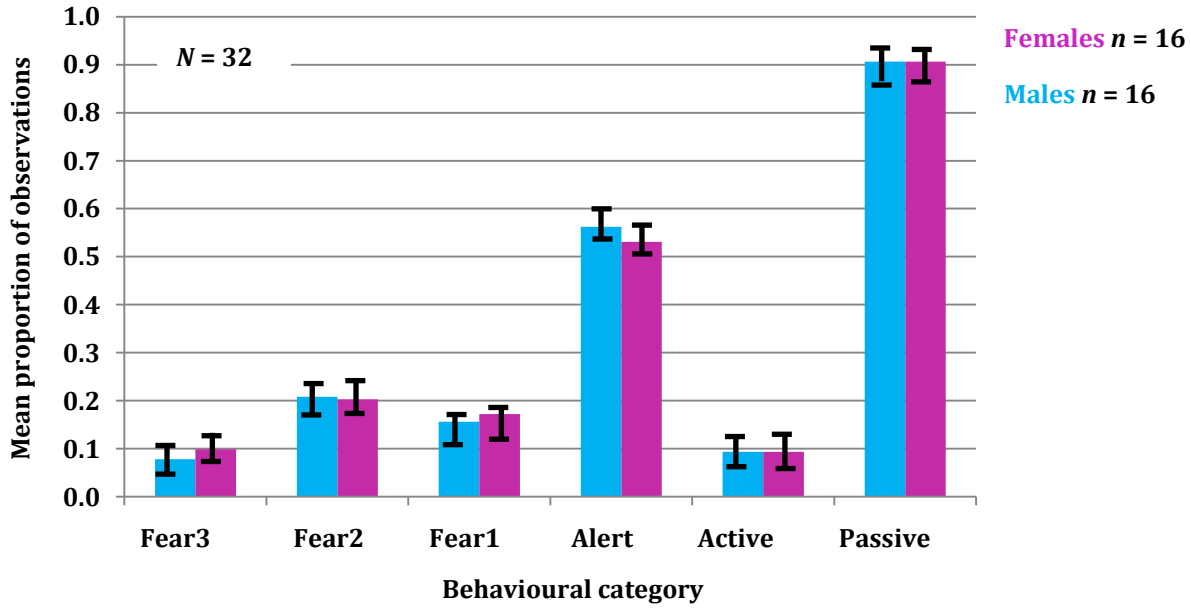
Males and females did not differ significantly in their responses during ECG and HDO recording (Figures 3.3.1 f & g).

Figures 3.3.1 f & g Mean behavioural responses of male and female macaques recorded during handling and restraint for baseline ECG (f) and HDO (g) measurements.

(f) ECG



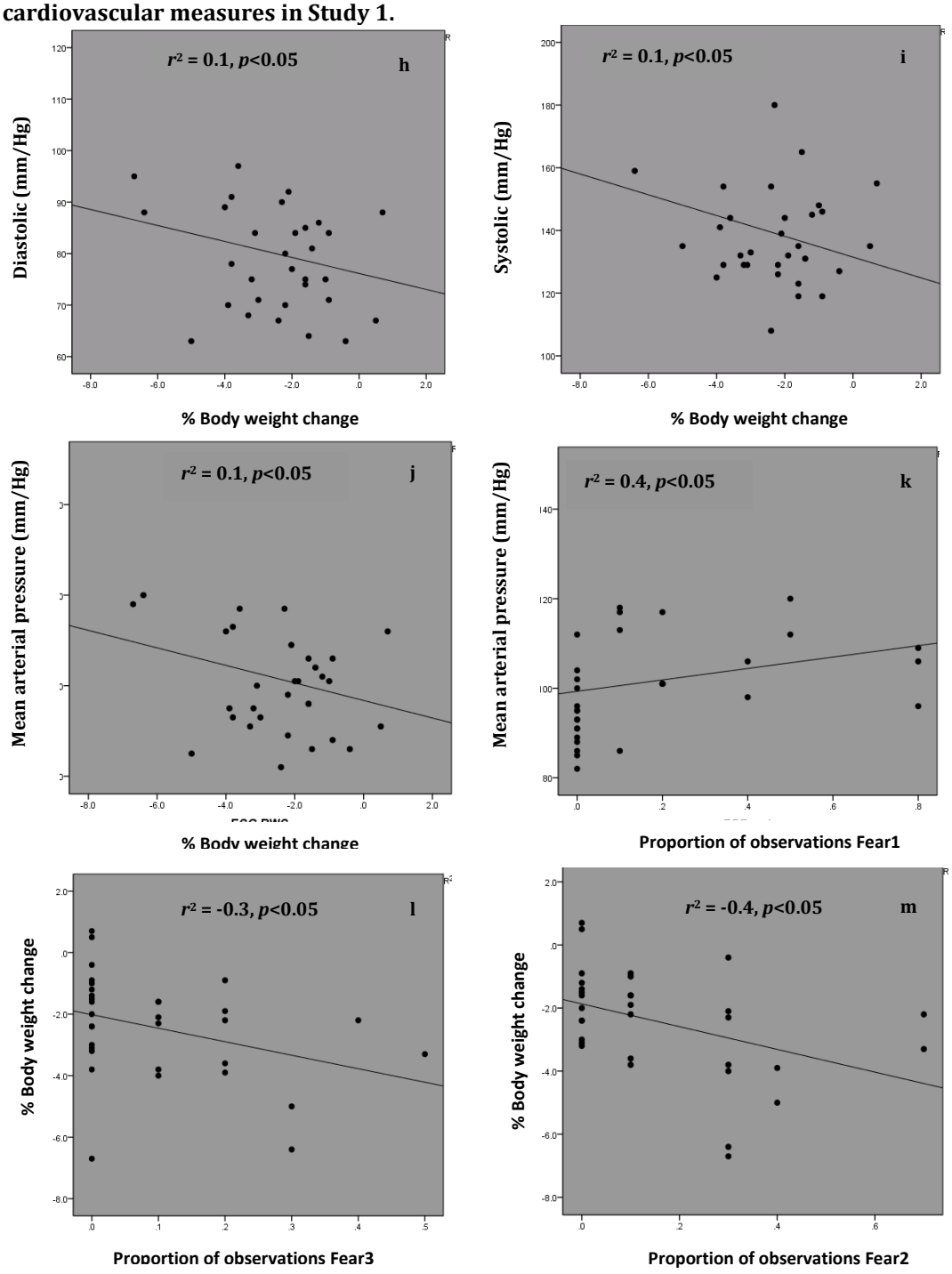
(g) HDO



Macaques were mostly alert and passive during recording of heart rates and blood pressures. There was a trend for animals to become more active and fearful with time in restraint tube during final blood pressure measures, but this was not found to be significant. No vocalizations were heard during recording.

Significant correlations of macaques' behavioural responses during ECG and HDO recording and physiological parameters are given in Figures 3.3.1 h – m.

Figures 3.3.1 h-m Correlations of macaque responses during ECG and HDO recording and cardiovascular measures in Study 1.



Plots h – j show a weakly negative correlation between percentage body weight change and blood pressure, indicating a trend for animals with greater weight loss having higher blood pressure. Furthermore, Fear1 facial expressions given during HDO recording are positively correlated with mean arterial pressure (k), and higher grade fearful expressions (e.g. Fear2 and Fear3) are negatively correlated with body weight change (l - m); more fearful animals experienced more weight loss.

3.3.2 Study (2)

3.3.2(1) Changes in body weight, body condition and alopecia during acclimatisation

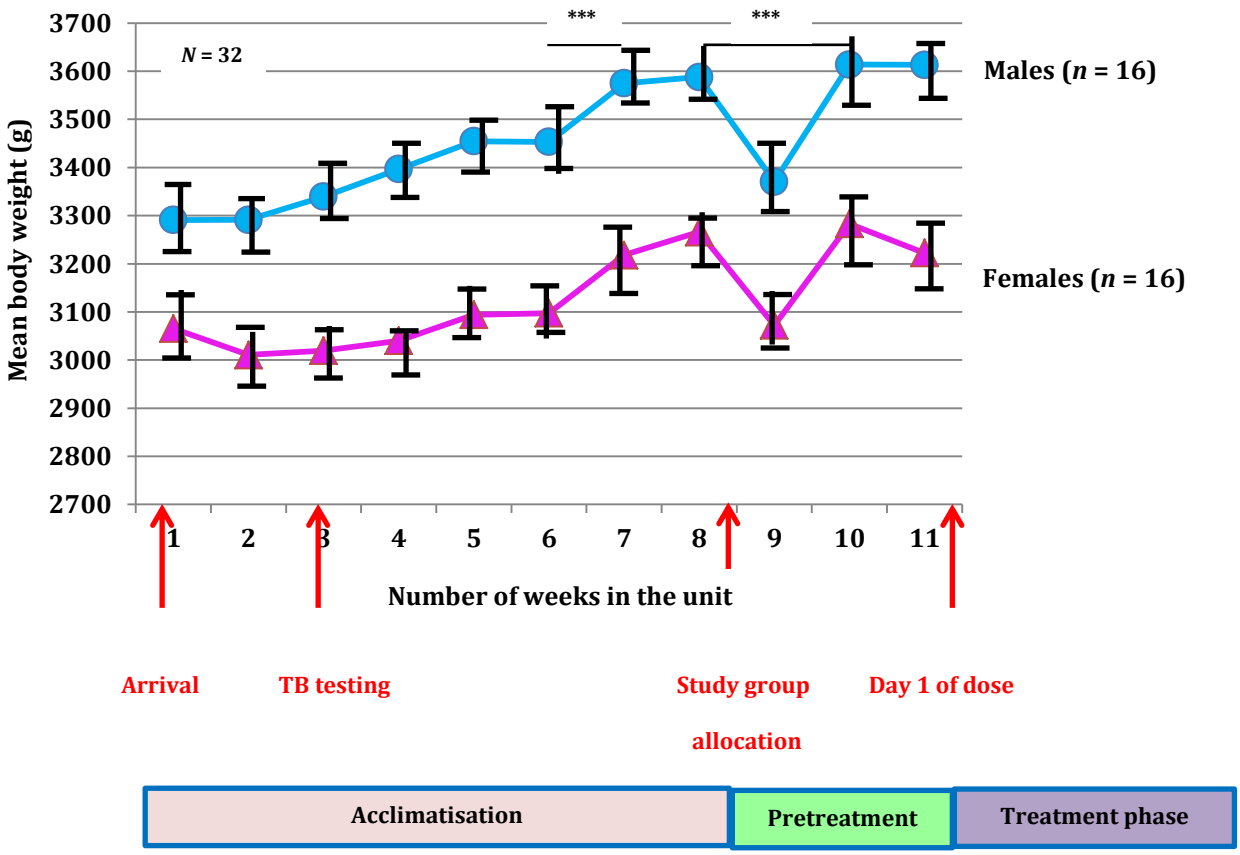
There were no significant differences in ages and body weights between Cohorts on arrival (p> 0.05). However, males were both older (Cohort A: $t(1) = 2.84$, $p<0.05$; Cohort B: $t(1) = 2.70$, $p<0.05$) and heavier than females (Cohort A: $f(10) = 10.19$, $p<0.001$; Cohort B $f(10) = 7.64$, $p<0.001$) and their body weight varied significantly with acclimatisation time and in response to procedures (Figures 3.3.2 a; Cohort A: $f(10) = 3.06$, $p<0.001$; Cohort B: $f(10) = 4.79$, $p<0.001$). Animals were heavier by week 10 than when they arrived (Cohort A $f(1) = 53.50$, $p<0.001$; Cohort B $f(1) = 29.71$, $p<0.001$), with Cohort A males showing a greater weight increase than males in Cohort B. Indeed the pattern of change during acclimatisation differed for both males and females and for each Cohort. For example Cohort B males experienced a significant drop in body weight between weeks 3 - 4 (3.2% $\pm$ 0.81; $f(1) = 18.72$, $p<0,005$); whilst a similar drop was seen in females a week later (3.1% $\pm$ 2.48; $f(1) = 67.67$, $p<0.001$). Cohort A male and female animals followed a more similar pattern of change to one another. They showed the greatest increase in body weight between weeks 6 - 7 (males: 3.8% $\pm$ 2.30, $f(1) = 8.57$, $p<0.001$; females: 4.8% $\pm$2.23, $f(1) = 6.15$, $p<0.005$), followed by a steep drop between weeks 8 - 9 (males: -6.9% $\pm$ 1.41; $f(1) = 8.83$, $p<0.05$; females: -5.5% $\pm$ 10.65, $f(1) = 8.33$, $p<0.005$). The latter difference coincided with a change in group composition that occurred in week 8; animals were transferred between pens because the study required treatment-free animals in groups 1and 4 (e.g. animals were moved from 4/pen to between 3-5/pen).

According to the clinical codes recorded by technicians, animals were in good physical health overall, with the exception of hair loss recorded in the same animals with an alopecia score of 2 or above. Significant decreases in body weight were not related to incidences of diarrhoea, dehydration, veterinary treatment etc. Indeed the only incidences of loose faeces were low (n = 3, females; n = 2, males) and observed in Cohort A animals during week 2.

Figures 3.3.2 a - c Fluctuation in body weight, body condition and alopecia scores during the acclimatisation and pretreatment period.

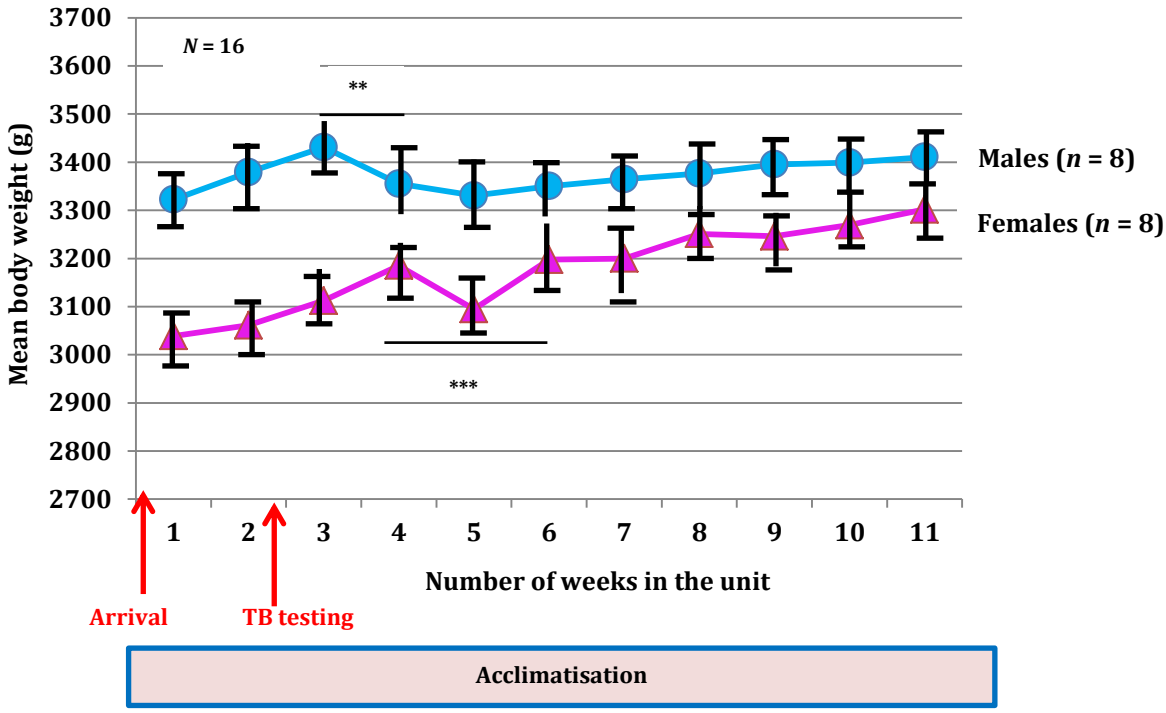
a) Body weight

Cohort A



Males and females displayed a similar pattern of body weight change week-to-week, with the greatest gains observed in week 6 – 7 and greatest fall between week 8 – 9 (males: mean - 6.9%; females: mean - 6.7%), which coincided with study group allocation at the start of pretreatment. The animals' body weight stabilized by week 10, start of dosing at study onset.

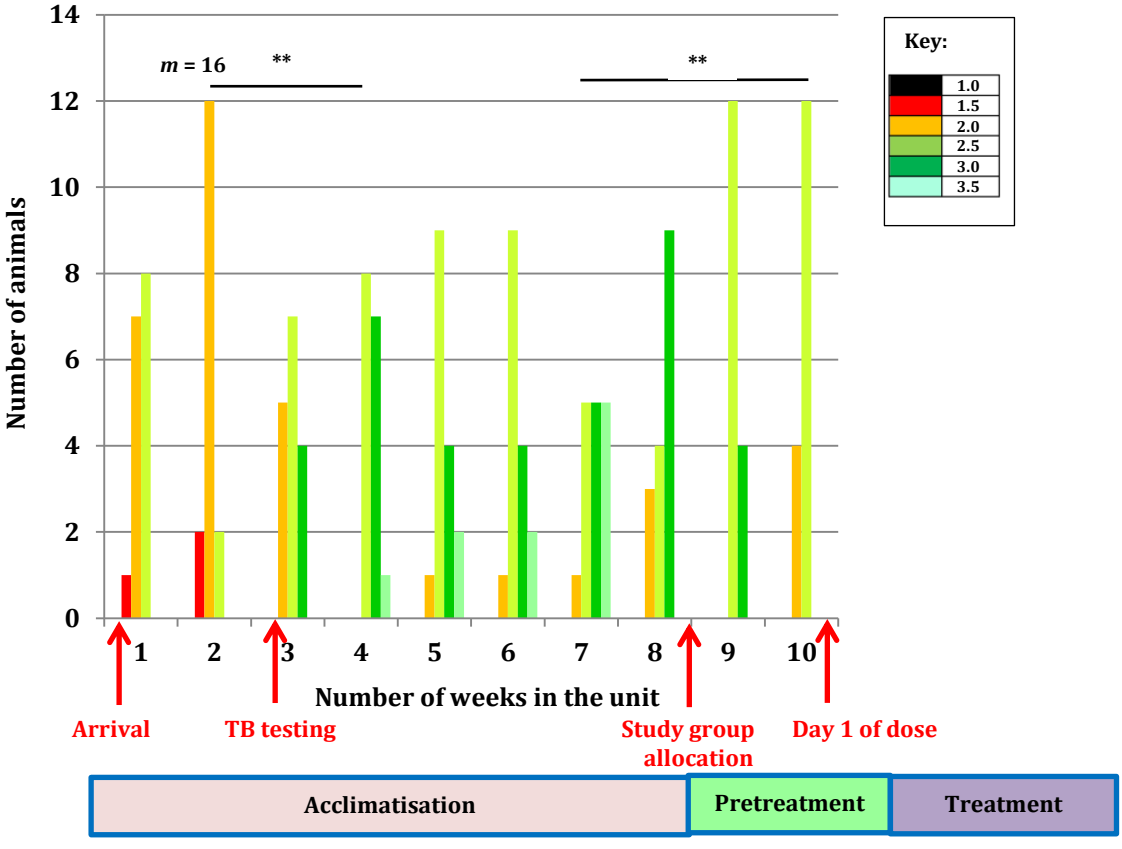
Cohort B



Cohort B animals followed a different pattern of body weight change compared to Cohort A. Males and females experienced the largest drop in body weight at different times; week 3 - 4 for males and week 4 - 5 in females.

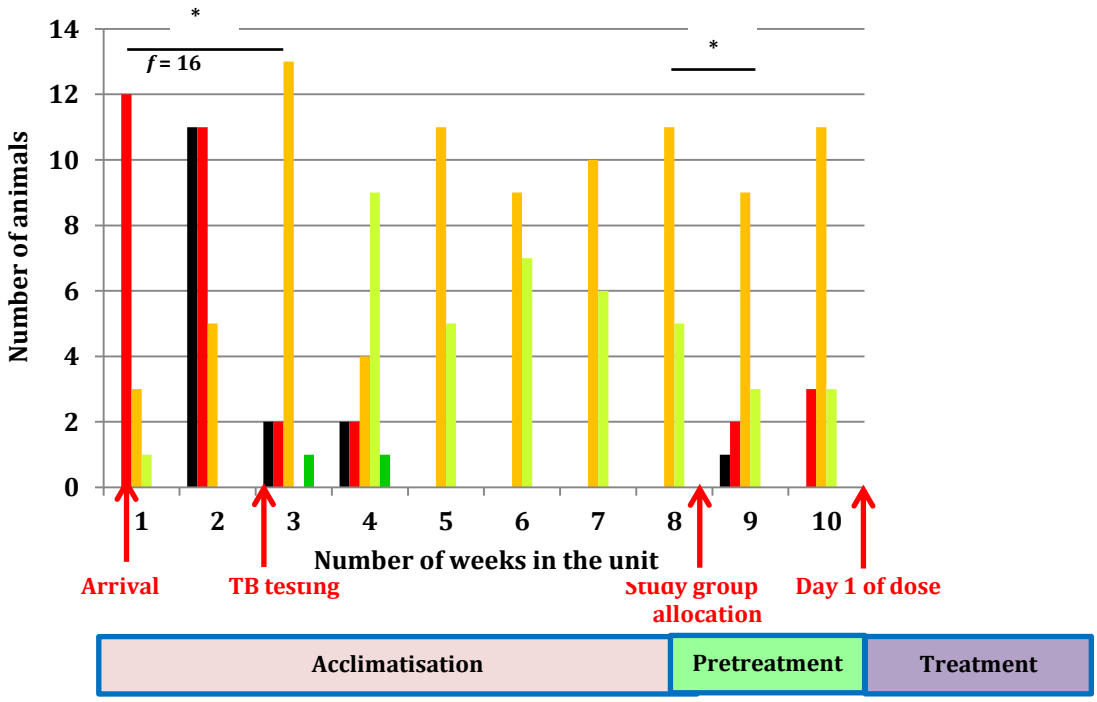
b) Body condition (Clingerman & Summers 2005)

Cohort A



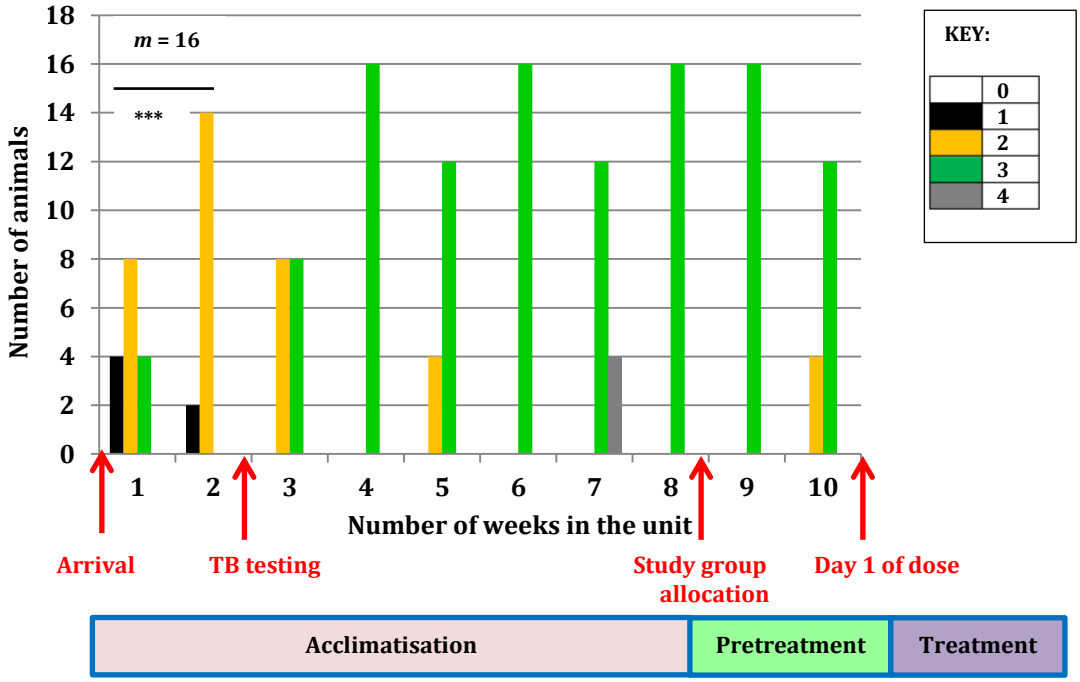
Males gained body condition over weeks 2 - 3 and 3 - 4, following the pattern of body weight increases. A drop in mean body condition score was recorded between weeks 8 - 9 and 9 - 10 as the number of animals with body condition scores 3.0 (ideal) fell to 2.5 (under weight) and 2.0 (thin). This was thought to coincide with weight loss during pretreatment. However, the pattern of change of body condition differed for males and females ($f(9) = 10.12$, $p<0.05$). Females entered the unit in poor body condition (e.g. 1.5; very thin) which worsened by week 2, improved in week 3, and followed a fluctuating but improving trend until pretreatment. Poor body condition, soon after arrival, was likely a result of non-significant weight loss and incidence of diarrhoea in week 2.

Cohort A



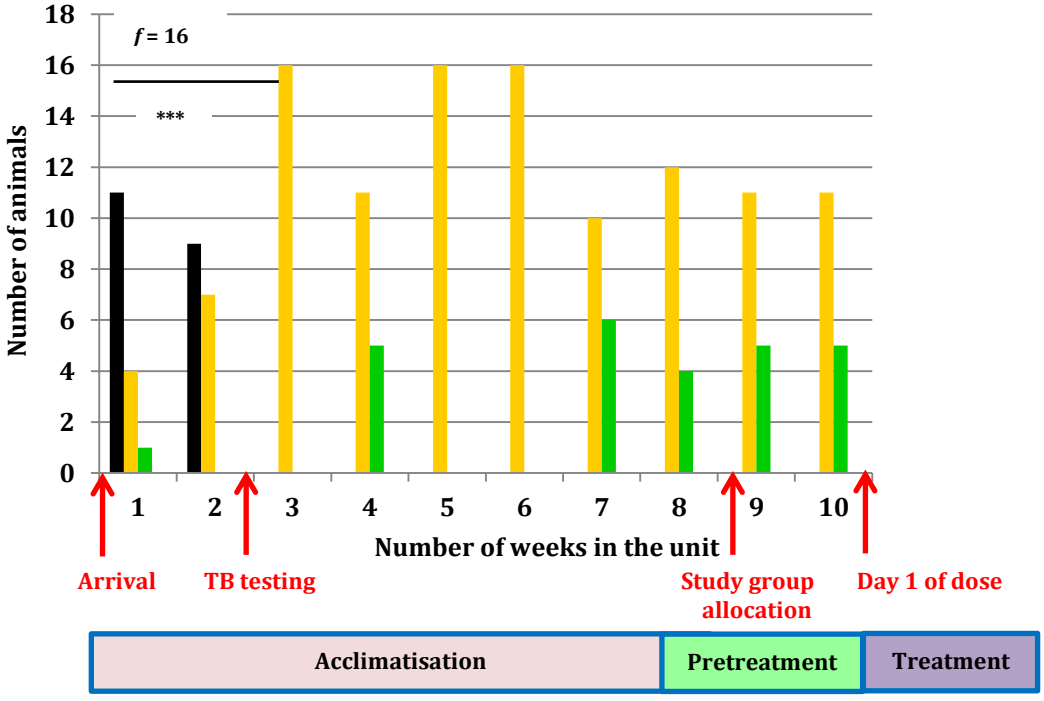
Body condition (Wolfensohn & Honess 2005)

Cohort A



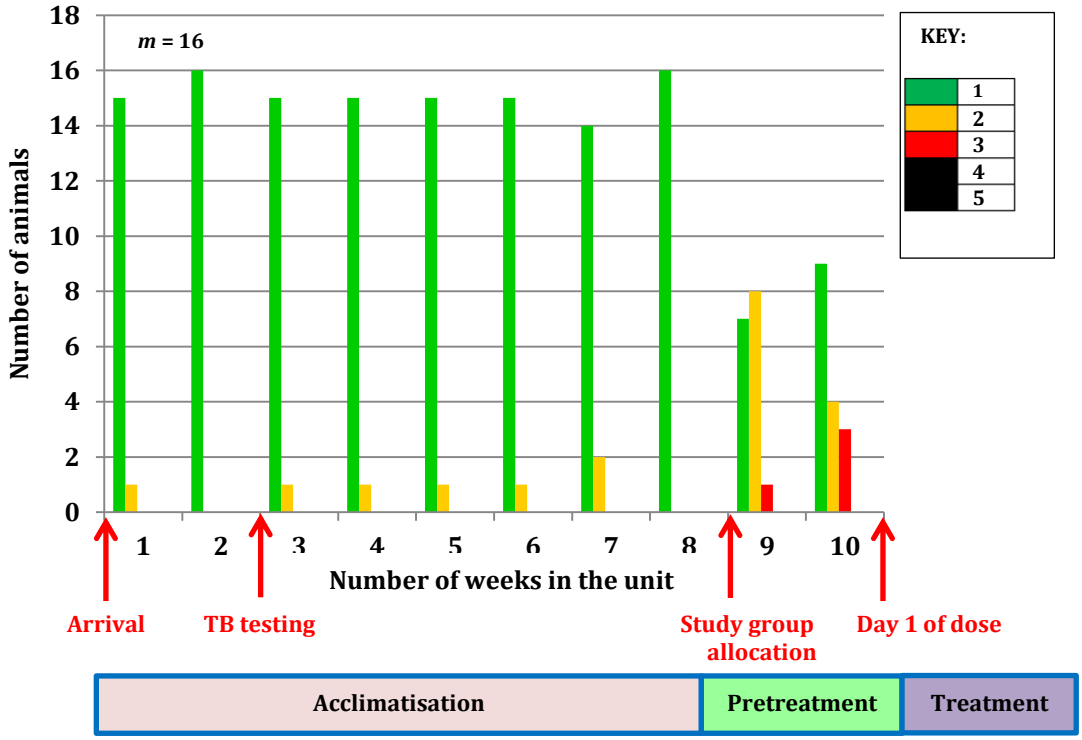
Using a different body condition scoring method (e.g. Wolfensohn & Honess 2005), scores were also found to vary with time in the unit, and between males and females. Differences were found in mean scores between weeks 1 – 2 (males: $f(1) = 34.59$, $p < 0.001$; females: $f(1) = 34.59$, $p < 0.001$) and 2 – 3 (males: $f(1) = 21.30$, $p<0.05$; females: $f(1) = 21.30$, $p < 0.05$). Changes reflect increasing body condition across weeks 1 – 3, following similar patterns in body weight. Females entered the unit with lower body condition scores (e.g. 1: severely underweight; 2: underweight), and males had a more normal body condition than females at start of pretreatment. Mean body condition did not drop significantly with baseline recordings.

Cohort A



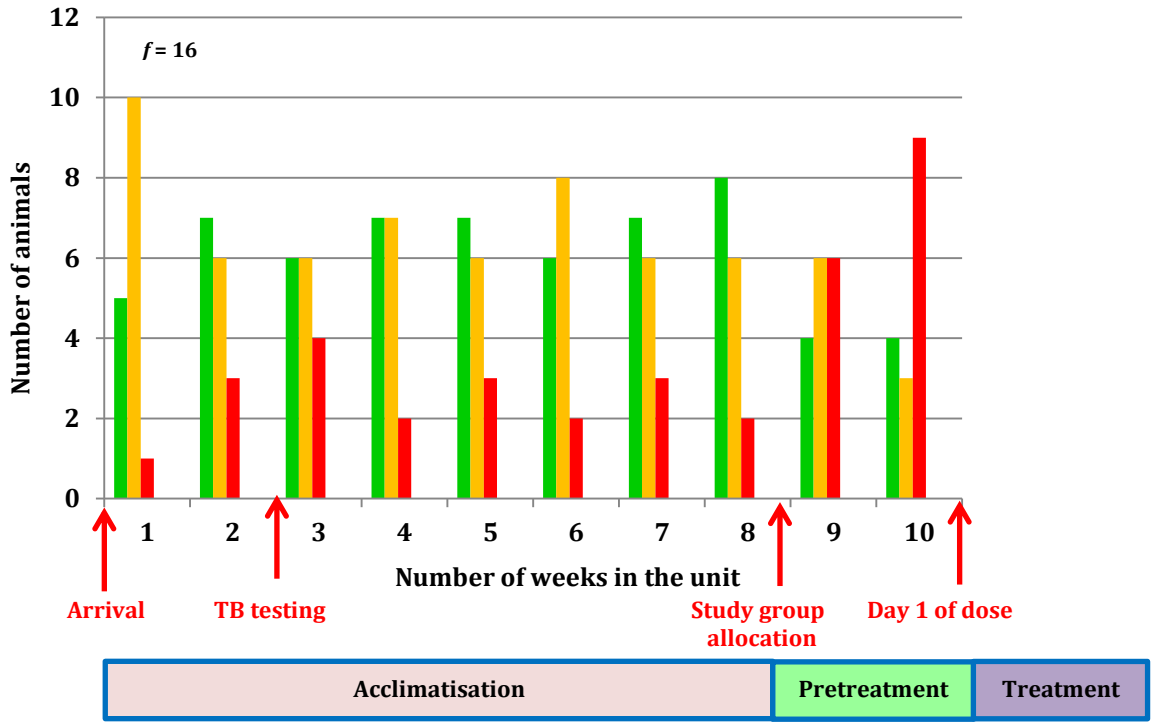
C) Alopecia scores (Honess *et al* 2005)

Cohort A



All but one male had normal pelage cover (1 = normal; 2 = small patches of hair loss; Honess *et al* 2005) up to week 8 acclimatisation. Following study group allocation, 9 animals (56.3%) developed alopecia; 8 males displayed small patches, whilst 1 animal showed greater hair loss (e.g. 3). This change was significant ($f(1) = 19.88$, $p < 0.001$). The pattern of change for females differed significantly to males ($f(9) = 5.85$, $p < 0.001$).

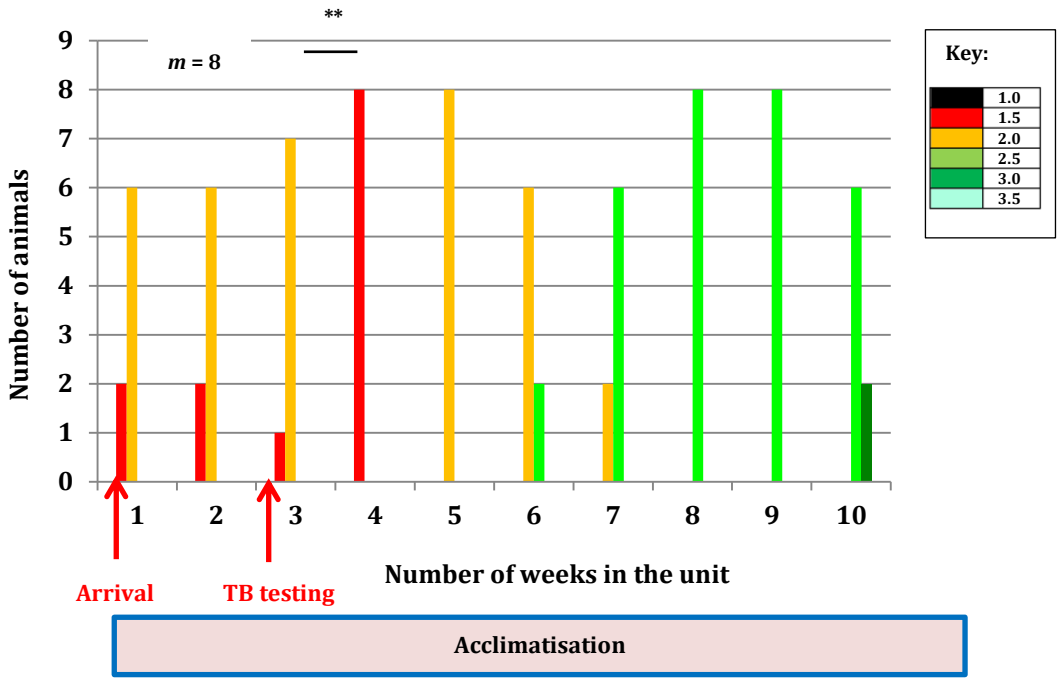
Cohort A



Eleven (68.8%) females entered the unit with a degree of hair loss; the majority (10; 62.5%) were small patches (e.g. 2). The severity of alopecia worsened with acclimatisation, by week 9 this was significant ($f(1) = 5.85$, $p<0.001$) with 12 (75%) females affected - 6 (37.5%) severely (e.g. 3). The majority of animals ($n = 9$; 56.3%) had grade 3 alopecia as they went on to study in week 10. Animals in Cohort B showed less severe alopecia over the acclimatisation period (Figure C: Cohort B males & females).

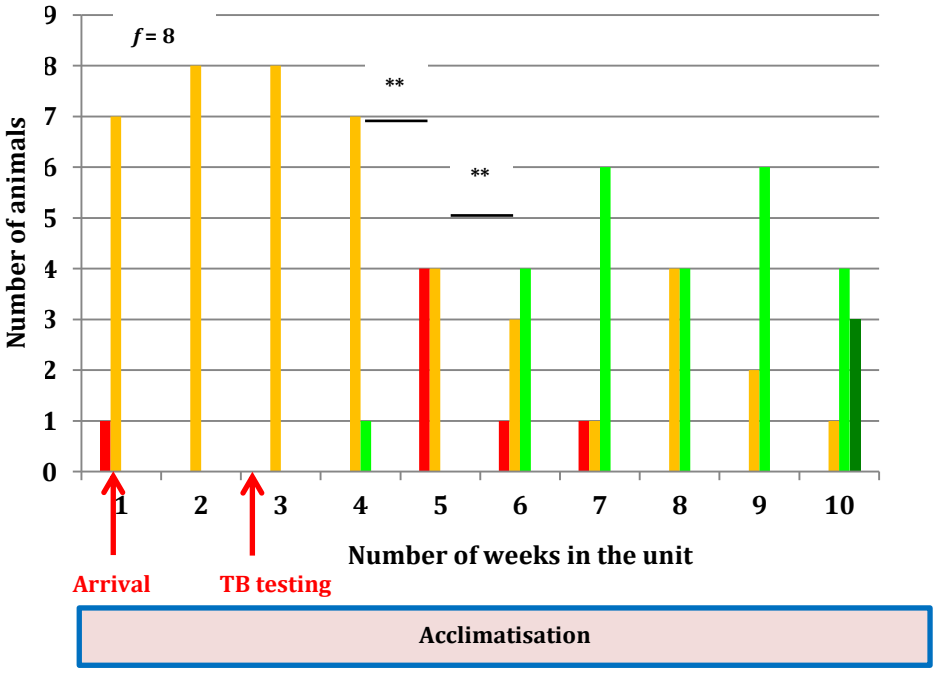
b) Body condition (Clingerman & Summers 2005)

Cohort B



There were significant differences over time in body condition scores ($f(9) = 37.26$, $p<0.01$,) and between males and females ($f(9) = 3.91$, $p< 0.001$).

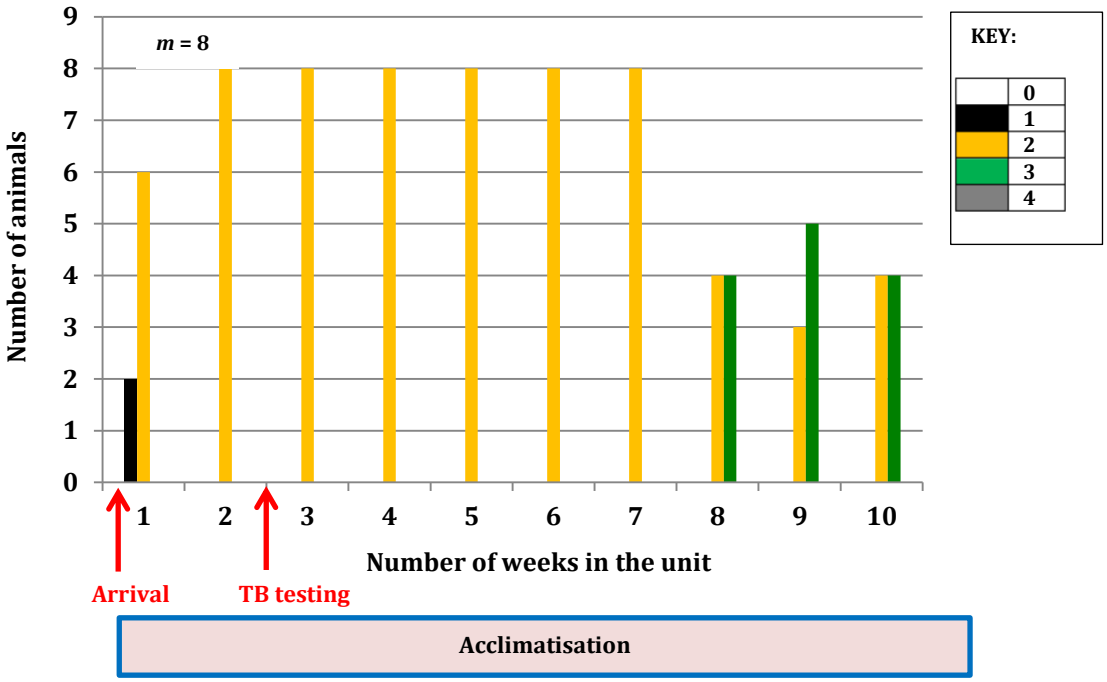
Cohort B



Mean body condition dropped significantly for males in weeks 3 – 4, following a similar pattern in body weight change (Figure 3.3.2). For females, body condition followed a similar rise and fall with body weight observed in weeks 4 - 5 and 5 – 6. The majority of males and females showed normal body condition by week 10 acclimatisation in the unit.

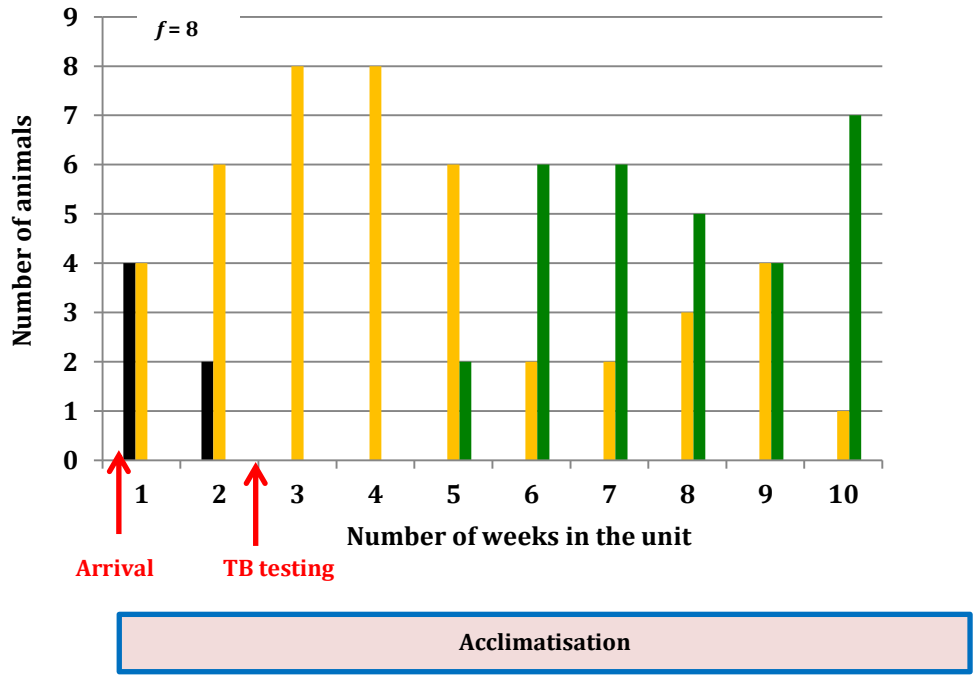
Body condition (Wolfensohn & Honess 2005)

Cohort B



Significant differences were recorded over time ($f(9)=20.23$, $p<0.05$) and between males and females ($f(9) = 5.42$, $p<0.05$).

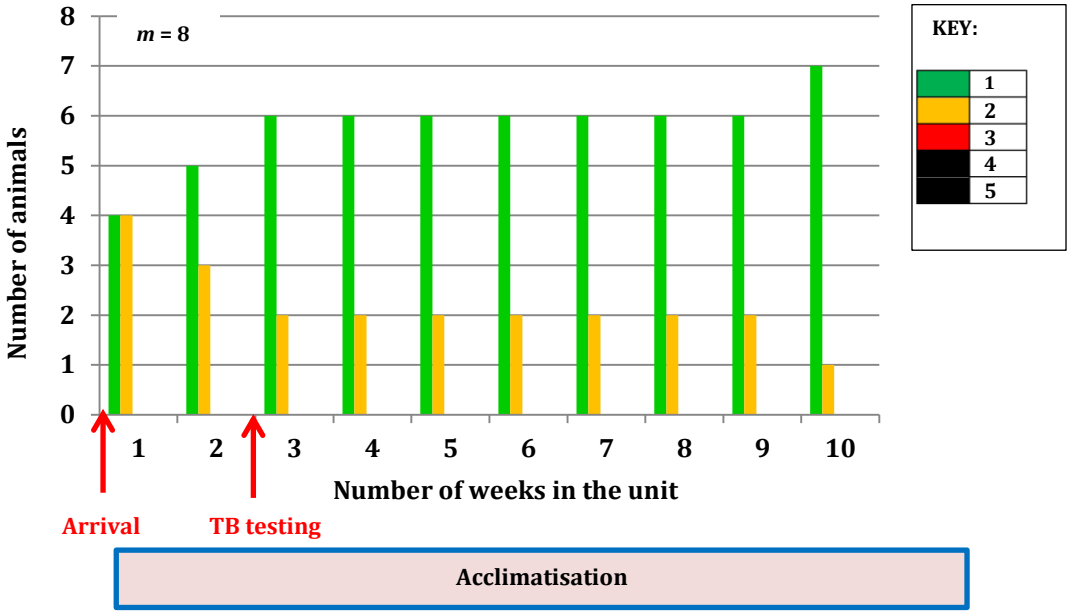
Cohort B



Using the Wolfensohn and Honess (2005) scoring method there appears to be a lag in body condition becoming normal (e.g. 3; week 7 vs. week 8) in males compared to scores awarded using Clingerman and Summers (2005). More females than males had a lower body condition on arrival, and more females (n = 7) than males (n = 4) were at normal body condition by week 10 on the unit.

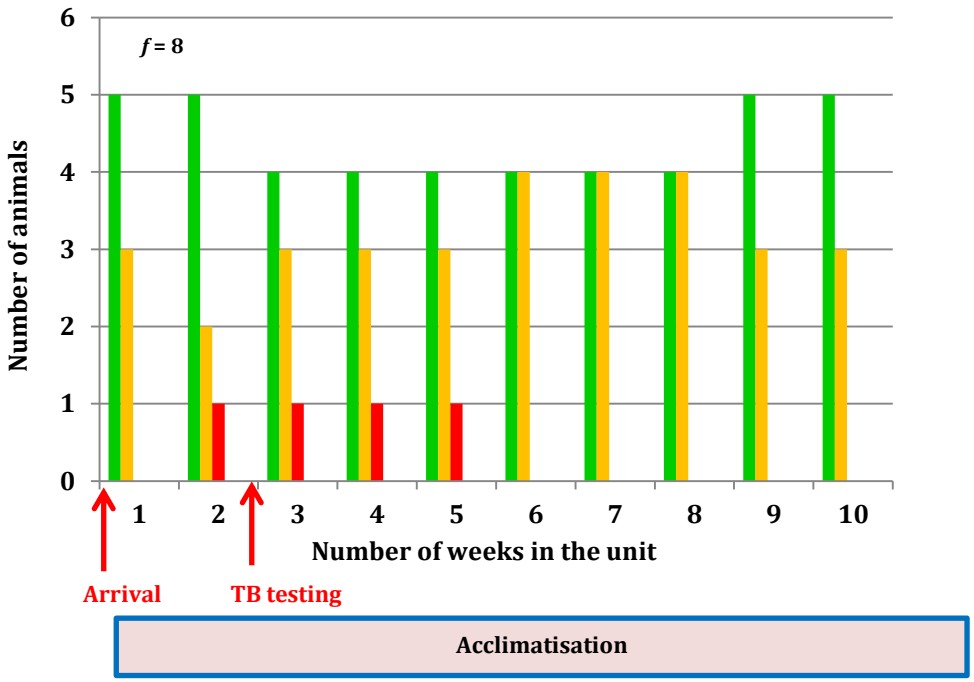
C) Alopecia scores (Honess *et al* 2005)

Cohort B



A proportion of both males and females in Cohort B entered the unit with small patches of alopecia (males: n = 4; 50%; females: n = 3; 37.5%). Severity of hair loss reduced over acclimatisation time, whereas hair loss in Cohort A animals, increased during weeks 8 – 10 with pretreatment.

Cohort B



3.3.2(2) Intra-observer reliability of body condition and alopecia scoring methods

The intra-observer reliability of body condition and alopecia scores from repeated scoring of the same animals during TB testing are given in Table 3.3.3 (2).

Table 3.3.3 a Intra-observer reliability of body condition and alopecia scoring methods

Score method	No of scores in agreement/disagreement (distance)	% agreement	Kappa coefficient
Body condition (Clingerman & Summers 2005)	43/5 (± 0.5)	89.6	0.84 (T^b 8.19, p<0.05)
Body condition (Wolfensohn & Honess 2005)	45/3 (± 1)	93.8	0.89 (T^b 7.65, p<0.05)
Alopecia (Honess *et al* 2005)	48/48 (0)	100	1.00 (T^b 8.26, p<0.05)

Distance: the magnitude of the disagreement between scores.

Agreement for all scores was high. Alopecia was the easiest to score even with a small amount of practice, as reflected in perfect agreement between pairs of scores given to the same animals. There were greater percentage agreements between pairs of scores using Wolfensohn & Honess (2005) body condition scoring system. This was not reflected in the kappa coefficient as the distance between disagreements was larger, because half-point measures are not used. Both body condition scoring methods could be used reliably in juvenile macaques with a small amount of training and experience.

3.3.2 (3) Relationship between macaque body condition scores and body weights

Body condition scores awarded using two different methods (e.g. Clingerman & Summers 2005; Wolfensohn & Honess 2005) correlated, moderately, positively with body weight (Clingerman & Summers 2005: $r^2 = 0.37$, $p<0.05$; Wolfensohn & Honess 2005: $r^2 = 0.38$, $p<0.05$).

3.3.2 (4) Relationship between multiple variables and cardiovascular parameters

Cardiovascular parameters

Male and female macaques in Cohort A did not have significantly different mean heart rate and blood pressures (Table 3.3.2 b).

Table 3.3.2 b Heart rate and blood pressure of macaques in Study (2)

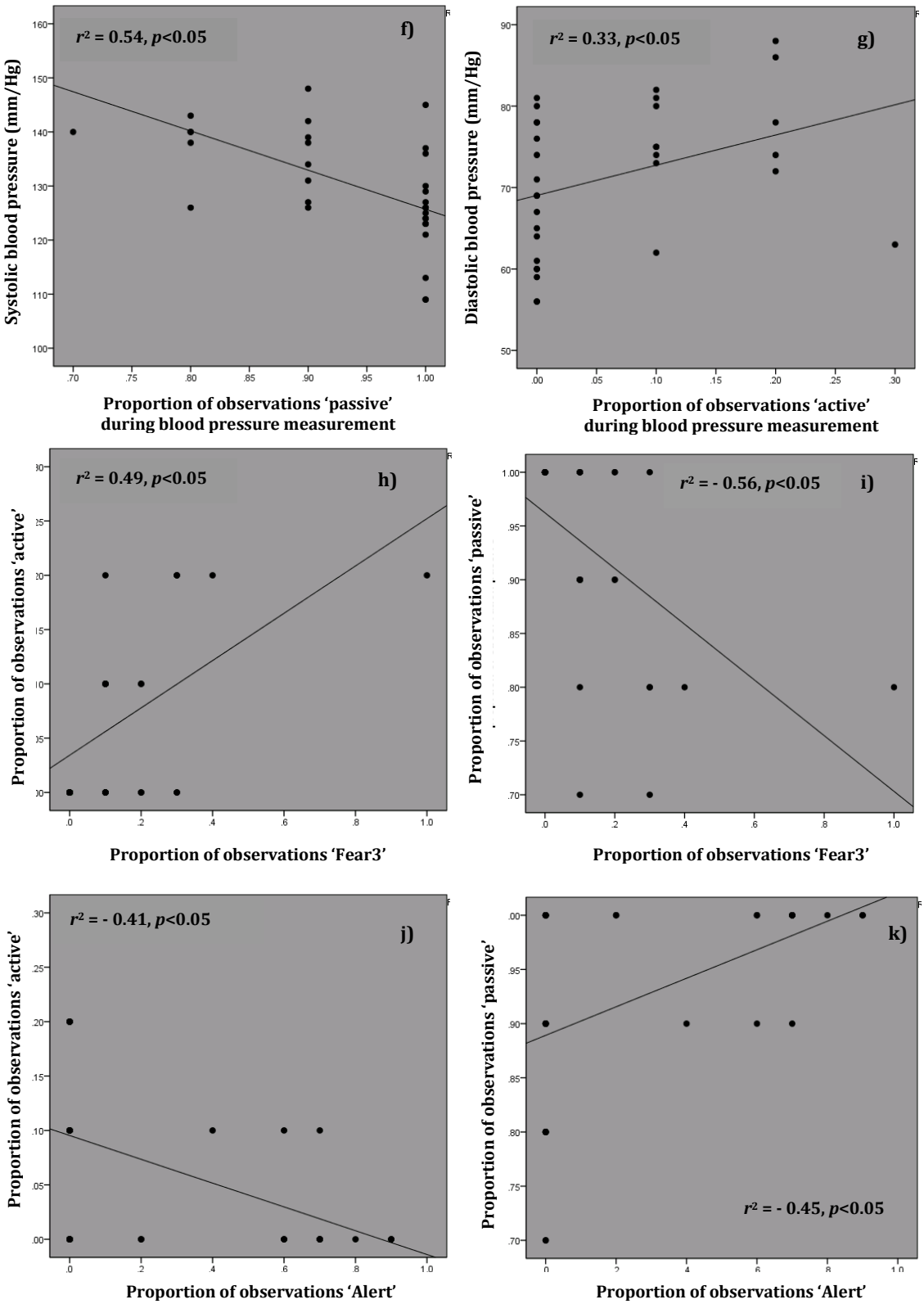
Sex	N	Cardiovascular measure	Mean (±SD)	O-R
Male	16	**Heart rate (bpm)**	219.3 (25.0)	157-237
		Systolic (mm/Hg)	132.1 (14.0)	109-157
		Diastolic (mm/Hg)	69.9 (10.2)	56-88
		Mean arterial pressure (mm/Hg)	91.1 (10.3)	75-106
		Pulse (bpm)	220.9 (25.1)	174-261
Female	16	**Heart rate (bpm)**	210.6 (25.4)	174-247
		Systolic (mm/Hg)	130.1 (25.4)	174-257
		Diastolic (mm/Hg)	73.0 (7.3)	59-82
		Mean arterial pressure (mm/Hg)	93.3 (6.2)	81-102
		Pulse (bpm)	232.6 (27.9)	193-269
All	32	**Heart rate (bpm)**	219.9 (25.2)	157-247
		Systolic (mm/Hg)	131.1 (10.9)	109-157
		Diastolic (mm/Hg)	71.5 (8.8)	56-88
		Mean arterial pressure (mm/Hg)	92.6 (8.4)	75-106
		Pulse (bpm)	226.8 (26.8)	174-269

N: Number of animals; **O-R:** Observed range (min-max). Cardiovascular measures did not differ significantly ($p>0.05$) between males and females.

Behavioural responses during ECG and HDO recording

As with cardiovascular parameters, male and female macaques did not significantly vary in their behavioural responses during recording. Five males and three females vocalised during recording, and this was always observed with Fear3 facial expression. Females emitted Khreet Screeches (median 2.5; range: 1.0 – 4.0), whilst males emitted Kra and Khreet Screeches in combination (Kra, median 2.0; range: 1.0 -3.0; Khreet Screech, median 3.0; range: 2.0 – 7.0). Significant correlates of behavioural responses and cardiovascular parameters are given in Figures 3.3.2 f – k.

Figures 3.3.2 f - k Correlation of behavioural responses and cardiovascular measures



There were no significant correlations between heart rate, mean arterial pressure, pulse, and behaviour during recording. Systolic and diastolic blood pressures display a reciprocal relationship with behaviour; systolic blood pressures were negatively correlated with being 'passive', whilst diastolic blood pressures were found to be positively correlated with 'active' behavioural responses. Unsurprisingly, high grade fear responses, Fear 3, were positively correlated with being 'active' and negatively correlated with being 'passive' during recording. Furthermore, displaying an 'alert' facial expression was negatively correlated with being active and positively correlated with passivity.

3.3.3 Study (3)

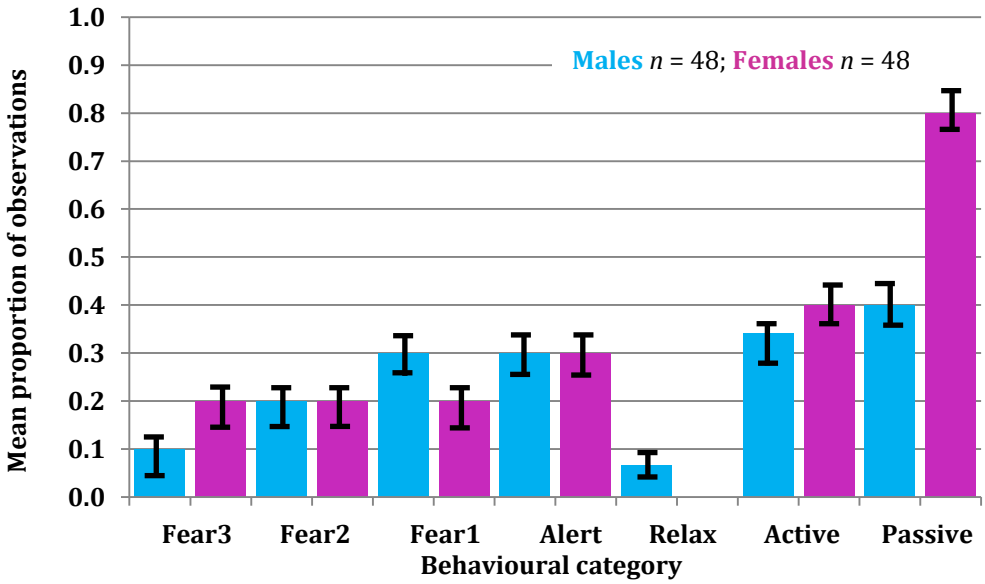
There were no significant differences in the age of macaques at start of study (p>0.05; Table 3.2.3 a). Macaques were in good health at start of pretreatment, during baseline recording.

3.3.3(1) Relationship between behavioural responses during restraint and heart rate, haematological and clinical chemistry parameters

Behavioural responses during recording

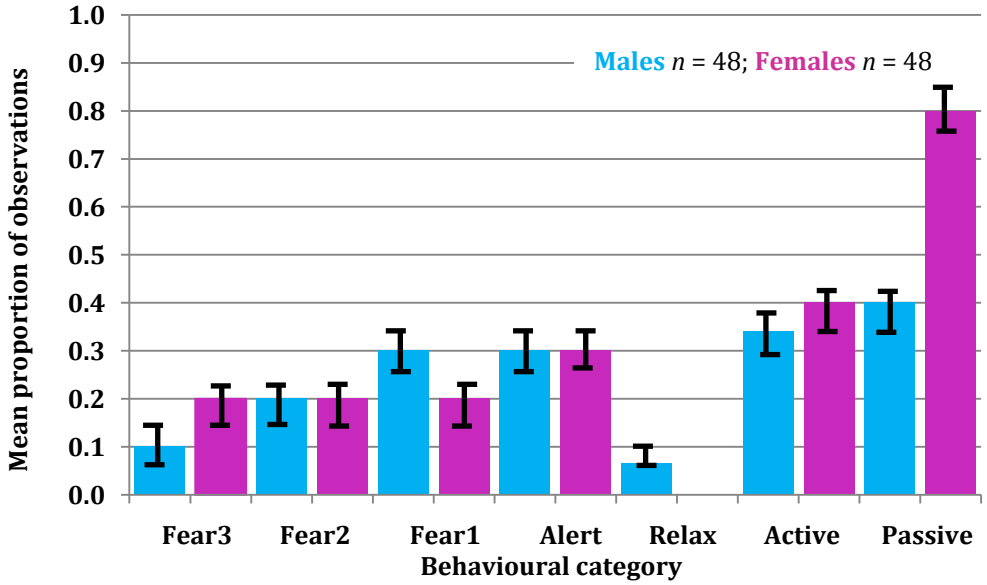
Behavioural responses (excluding vocalisations) of males and females, and between Cohorts were not significantly different; all data were therefore pooled and entered into a regression model. The mean behavioural responses of males and females, pooled for Cohort are displayed in Figures 3.3.3 a - b. Vocalisations were only recorded from animals in Cohort B, with calls; Kra, Wraagh and Khreet Screech emitted in low frequencies (max 3 vocalisations recorded per animal per 30 second recording). They accompanied Fear3 and showed colinearity, and were therefore not included as a 'predictor variable' in the regression model (Table 3.3.3 b). No vocalisations were given during venepuncture.

Figure 3.3.3 a Mean behavioural responses of males and females during restraint for ECG recording



Macaques were most often displaying Fear1 or alert facial expressions, and females were more passive than males during ECG recording.

Figure 3.3.3 b Mean behavioural responses of males and females during restraint for venepuncture



Macaques showed similar behavioural response to venepuncture as during ECG recording.

Physiological responses

The physiological parameters of male and female macaques recorded at baseline for Study (3) are given in Table 3.3.3 a. Gender was found to have a significant effect on six outcome variables in the regression model (e.g. neutrophils: N, lymphocytes: L, alkaline phosphatase: ALP, gamma glutamyltransferase: gamma GgT, heart rate and body weight change; Table 3.3.3 a).

Table 3.3.3 a Mean physiological data for male and female cynomolgus macaques in Study (3)

Outcome variable	Unit	All		Male		Female	
		Mean (±SD)	O-R	Mean (±SD)	O-R	Mean (±SD)	O-R
Haematology							
Hb	g/dL	13.5 (0.76)	11.9-15.7	13.6 (0.82)	12.0-15.7	13.0 (0.67)	11.9-14.8
RBC	mil/ cmm	6.88 (0.48)	5.72-8.41	6.87 (0.48)	5.72-8.41	6.88 (0.49)	5.78-8.12
PCV	%	44.5 (94.39)	38.9-50.1	44.6 (2.89)	39.3-50.1	44.4 (102.30)	38.9-49.1
PLAT	1000/ cmm	402 (94.39)	183-587	402 (86.87)	233-587	402 (102.30)	183-552
WBC	10^9/L	14.7 (3.29)	6.5-22.9	14.8 (3.12)	9.4-19.8	14.7 (3.48)	6.5-22.9
N	10^9/L	8.3 (2.68)	3.2-16.3	7.7 (1.95)	4.3-11.6	9.0 (3.15)	3.2-16.3
L	10^9/L	5.6 (1.61)	3.0-9.9	6.3 (1.70)	3.7-9.9	4.9 (1.19)	3.0-7.4
Clinical chemistry							
AST	IU/L	34 (10.25)	24-112	35 (13.10)	24-112	34 (6.39)	24-45
ALT	IU/L	36 (12.52)	15-76	36 (13.90)	15-76	36 (11.11)	17-62
ALP	IU/L	568 (199.35)	295-1528	638 (244.53)	373-1528	498 (102.52)	295-682
GgT	IU/L	126 (30.46)	75-235	143 (30.90)	75-235	108 (17.40)	77-148
UREA	mmol/L	5.6 (1.45)	3.5-13.1	6.0 (1.72)	4.1-13.1	5.2 (0.98)	3.5-7.6
TBILI	mmol/L	3.1 (1.21)	1.7-6.2	2.9 (1.04)	1.9-6.2	3.3 (1.85)	1.7-6.0
CREAT	mmol/L	70 (10.02)	49-93	71 (10.18)	49-93	68 (9.75)	53-90
TPROT	g/L	88 (3.86)	78-99	88 (3.73)	78- 95	87 (4.01)	79-99
GLOB	g/L	40 (3.3)	30-49	39 (3.05)	30-45	41 (3.27)	36-49
HR							
HR	bpm	224 (26.01)	149-285	216 (27.46)	149-260	223 (21.44)	162-285
BWC							
BWC	%	-1.9 (2.71)	-12.1-3.2	-0.9 (1.32)	-4.0-1.7	-2.8 (3.33)	-12.1-3.2

OR: Observed range of values (minimum-maximum). Haematological and clinical chemistry analytes are defined in Appendix 1.1.

Table 3.3.3 b summarises the regression model for Study 3. The variation in only eight outcome (physiological) measures could be accounted for by the chosen predictors (Table 3.2.Study 3). In four of the physiological measures (e.g. PLAT, N, L and HR), macaque behavioural responses were found to be having a significant but varying effect (range: 13.2 – 83.4%), with the greatest effect (83.4%) observed on heart rates during ECG recording. Habituation was not found to account for variation in any of the physiological outcome measures.

Figures 3.3.3 c – j, display significant correlations between individual behavioural parameters during recording and physiological outcomes at baseline.

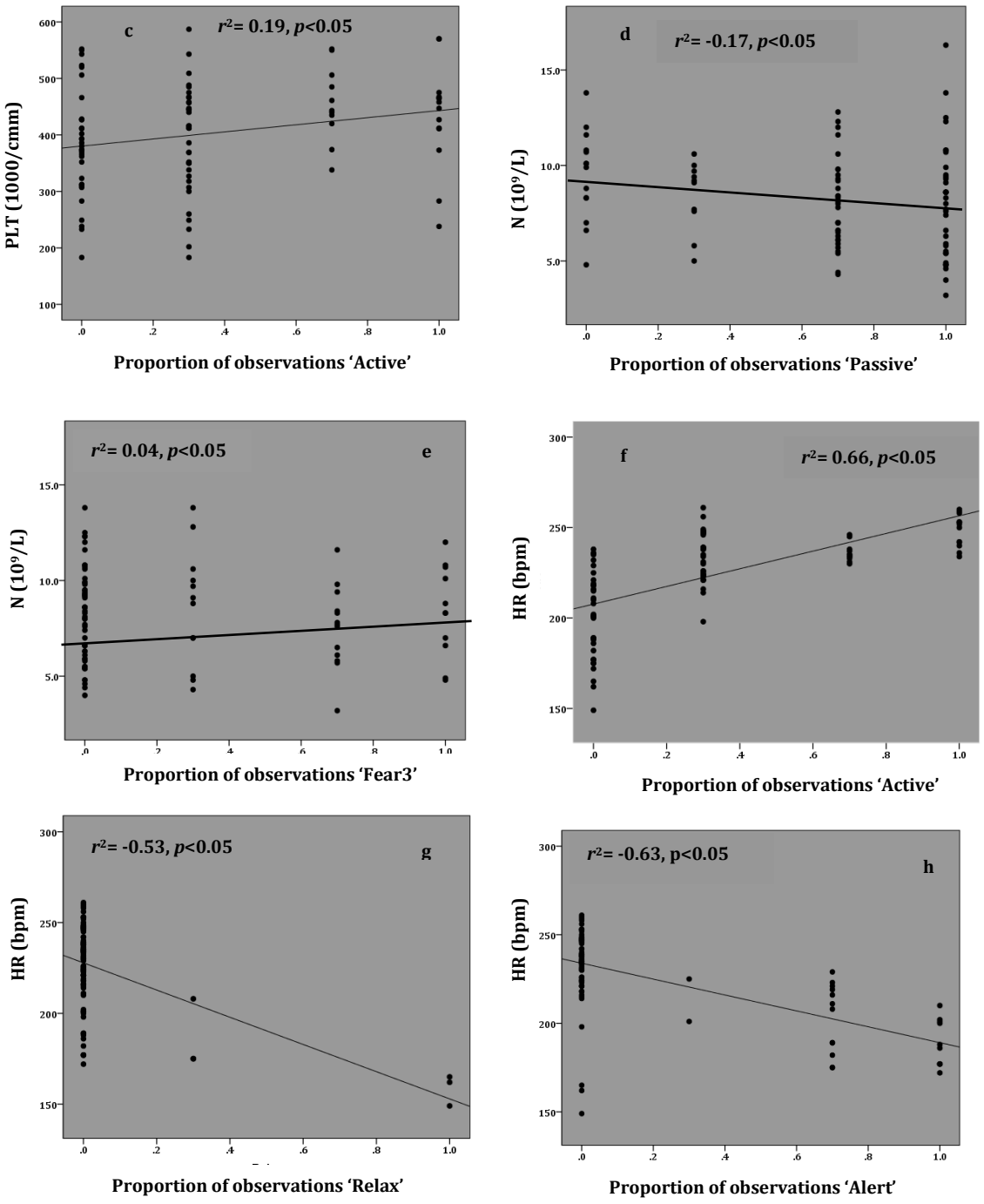
Table 3.3.3 b Regression model output for Study (3)

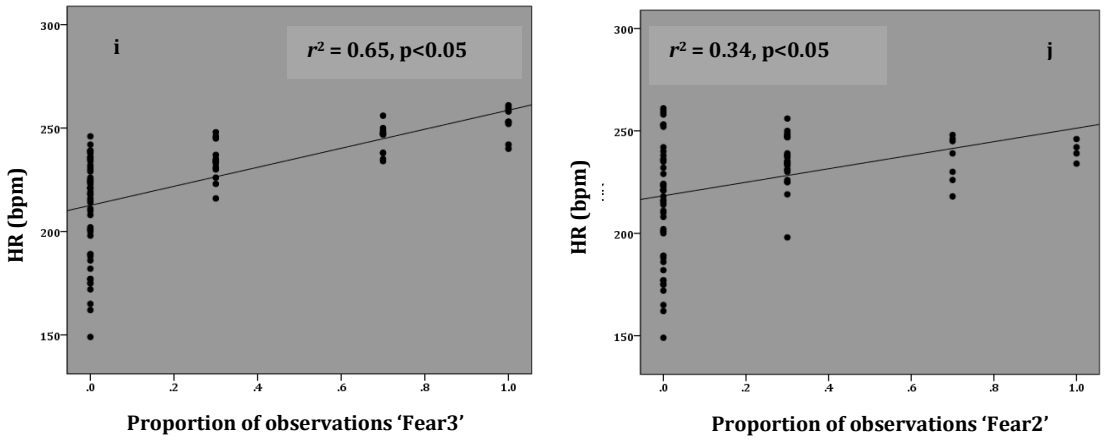
Biological (outcome Variable)	Predictors	Model summary		Model parameters			Significance of model (ANOVA)	
		R^2	%	B	SE	β	df	F
Haematology								
Hb (g/dL)	Constant Age	 0.04	 4.0	15.23 -0.00	0.87 0.00	 -0.21*	1	4.09*
Overall model *$R^2(1)=0.04$*;4%*								
RBC (mil/cmm)	**0 variables were entered into the model by SPSS**							
PLAT (1000/cmm)	Constant Active	 0.24*	 23.6	380.28 62.88	13.50 27.33	 0.24*	1	5.29*
Overall model *$R^2(1)=0.24$*;23.6%*								
PCV (%)	**0 variables were entered into the model by SPSS**							
WBC (10^9/L)	**0 variables were entered into the model by SPSS**							
N (10^9/L)	Constant Sex Passive Fear3	- 0.06** 0.10* 0.19**	- 6.0 4.2 9.0	8.76 1.73 -3.40 2.92	1.04 0.53 0.94 0.94	- 0.33*** -0.45*** 0.70**	3	6.93***
Overall model *$R^2(3)=0.19$*;19.1%***								
L (10^9/L)	Constant Sex Relax	 0.18*** -0.22*	 17.9 3.7	7.67 -1.30 1.67	0.49 0.30 0.83	 -0.40 0.19	3	12.27***
Overall model *$R^2(2)=0.22$*;21.6%*								
Clinical chemistry								
AST (IU/L)	**0 variables were entered into the model by SPSS**							
ALT (IU/L)	**0 variables were entered into the model by SPSS**							
ALP (IU/L)	Constant Sex	 0.13**	 12.5	778.59 -140.28	61.81 39.09	 -0.35**	1	12.88**
Overall model *$R^2(1)=0.13$*;12.5%*								
GgT (IU/L)	Constant Sex	 0.33***	 33.0	177.78 -34.78	8.27 5.23	 -0.57**	1	44.25***
Overall model *$R^2(1)=0.33$*;33.0%*								
UREA (mmol/L)	**0 variables were entered into the model by SPSS**							
TBILI (mmol/L)	**0 variables were entered into the model by SPSS**							
CREAT (mmol/L)	**0 variables were entered into the model by SPSS**							

TPROT (g/L)	**0 variables were entered into the model by SPSS**							
GLOB (g/L)	**0 variables were entered into the model by SPSS**							
Heart rate								
HR (bpm)	Constant			210.20	4.23	-	6	86.31***
	Active	0.44***	44.1	6.53	4.69	0.09*		
	Relax	0.59***	15.3	-60.76	6.17	-0.43***		
	Alert	0.75***	16.0	-29.21	3.78	-0.41***		
	Fear3	0.82***	7.0	23.91	4.20	0.34***		
	Sex	0.85***	2.6	8.31	2.22	0.16***		
	Fear2	0.86*	1.0	10.86	4.51	0.11**		
Overall model *R^2(6)=0.86*;85.9%***								
Body weight change								
BWC (%)	Constant			8.90	3.08		1	10.72***
	Sex	0.13***	13.1	-2.09	0.52	-0.39***		
	Age	0.19**	6.3	-0.01	0.00	-0.25*		
Overall model *R^2(1)=0.19*;19.0%*								

Data given to two decimal places. R^2 (*df*) is a measure of how much variability in the biological (outcome) variable is accounted for by the predictors selected by SPSS to be included in the model. The significance of the model at predicting the biological (outcome) variable is determined in the model using ANOVA. **p*<0.05, ***p*<0.01, and ****p*<0.001; Correlation coefficients: *r* = 0.10 small effect; *r* = 0.30 medium effect; *r* = 0.50 large effect (Field 2005, p32).

Figures 3.3.3 c - j Correlation of physiological parameters and behavioural responses during recording in Study 3.





During venepuncture, being 'Active', 'Passive' or displaying 'Fear3' facial expression were found to have weak correlations with haematological parameters. 'Active' is positively correlated with platelet count (c), whereas passivity (d) is negatively correlated with neutrophil count, and Fear3 (e) is positively correlated. Heart rate was strongly positively correlated with being 'Active' (f), and displaying Fear3 (i) or Fear2 (j) during recording. Conversely showing 'Alert' (h) or 'Relax' (g) behavioural expressions during recording were negatively correlated with heart rate.

3.3.4 Summary of results from Studies 1-3

Mean body weight was found to decrease during pretreatment procedures in Studies 2 and 3. It remained constant in Study 1. Nonetheless, animals in the first study showed less weight gain overall during acclimatisation compared to animals in Study 2, and assessment of body condition would have been a useful adjunct to determine if changes in body fat or muscle were occurring in response to pretreatment procedures. Furthermore animals in Study 1 showed similar behavioural responses during restraint and had marginally higher heart rates and blood pressures, indicating they found the experience just as stressful as animals in Study 2 and 3. In addition, in animals that lost weight, greatest weight loss correlated with higher blood pressures (e.g. systolic, diastolic and mean arterial) and frequency of Fear2 and Fear3 facial expressions given during HDO recording. Macaques in Study 2 showed the greatest percentage weight loss (males:-6.9%; females:6.7%) out of all three studies, and this coincided with both regrouping and pretreatment procedures. Regrouping prior to study was not undertaken in Studies 1 or 3.

Body condition tracked changes in body weight and decreased over the pretreatment period (Study 2). Indeed both condition scoring methods (e.g. Clingerman & Summers 2005; Wolfensohn & Honess 2005) produced scores that positively correlated with body weight and both were found to be reliable. However, the Clingerman and Summers (2005) method, which includes half-point

scores, was more sensitive to changes in juvenile macaques in response to pretreatment procedures. For this reason it was included in the welfare assessment framework (Table 3.4.1) and used in Chapter 5 to evaluate planned Refinement.

Incidence and severity of hair loss worsened following regrouping of animals and pretreatment procedures (Study 2). The pattern of hair loss was likely to be exacerbated by social stress experienced with changes in study group composition (see discussion below). However, in the absence of behavioural observations to confirm this hypothesis, this finding should be interpreted with caution.

Study 1 found macaques became less fearful during handling with increasing time spent on the unit (e.g. acclimatisation). However, during post – handling observations, animals consistently spent more time huddling, embracing, in contact, visually scanning the door from highest vantage points in the pen, and as a consequence their behavioural repertoire narrowed. Nonetheless with acclimatisation macaques showed a shift towards a more varied repertoire in baseline, pre – handling condition, with increased time spent playing, exploring, foraging/feeding and relaxed locomotion. I also observed that they spent less time auto- and allo-grooming in week 7 compared to earlier observations in week 4. Furthermore, in week 7, following handling, animals showed a faster recovery, as indicated by significant differences between behaviour observed in hour 1 and hour 2. For example, they spent less time inactive alert and embracing, and more time exploring and foraging/ feeding. Nonetheless despite this significant change macaques' behavioural responses did not reach baseline levels by the end of my observations, 2h+ after weighing and clinical observations, and they showed significant alterations in subsequent night-time behaviours.

Macaques spent less time asleep before and after midnight following handling and this correlated positively with Wraagh vocalisations given during physical examination and weighing. Wraagh vocalisations were also found to positively correlate with being awake or active before midnight. Moreover in Week 4 on the unit the percentage of time inactive alert following handling negatively correlated with Fear2 and passive responses, and positively correlated with being active during physical examination. The higher levels of auto-grooming observed following handling in week 4

were positively correlated with Khreet Screeches given. Similar patterns were not observed in week 7, but play, exploration, relaxed locomotion were found to negatively correlate with Fear1 facial expressions during handling and agitated locomotion correlated positively with Wraagh vocalisations.

Body shaking was observed infrequently and self-scratching was difficult to record accurately when animals were huddled together, they were therefore excluded from analysis.

Heart rates varied widely between macaques (range: 149 – 285 bpm across Studies 1 - 3) but mean heart rates recorded in each Study were fairly similar (e.g. Study 1: 222.3 bpm; Study 2: 219.9 bpm; Study 3: 224.0 bpm), and males and females did not differ significantly. Likewise blood pressures were similar in males and females, but highly variable between animals (range: systolic: 108 – 197; diastolic: 56 – 116; mean arterial: 75 – 144). Study 1 animals had higher mean blood pressures than those recorded in Study 2 (Study 1: systolic 139.6, diastolic 79.9, mean arterial 101.5; Study 2: systolic 131.1, diastolic 71.5, mean arterial 92.6). In Studies 1 and 2 behavioural responses during recording correlated with blood pressures but this differed between studies. Proportion of Fear1 positively correlated with mean arterial pressures in Study 1. Being active or passive showed a reciprocal relationship with systolic and diastolic pressures in Study 2. Passive responses negatively correlated with systolic pressure whereas active responses correlated positively with diastolic measures. Likewise in Studies 1 and 2, heart rate did not correlate with behavioural responses during recording. Yet in Study 3, Active, Fear3, Fear2, relaxed and alert facial expressions were strongly correlated with heart rate. Relaxed and alert expressions negatively correlated, whilst Active, Fear3 and Fear2 did so positively.

Study 3 also found platelets and neutrophil counts to be weakly correlated with behavioural responses. Platelet counts were positively correlated with being active, and neutrophils were negatively correlated with being passive, but positively correlated with Fear3.

3.4 Discussion

Data reported in this Chapter are recorded from naïve, juvenile and young adult cynomolgus macaques during acclimatisation and baseline recording for regulatory toxicology. Animals were in good health unless indicated, and were examined by a vet before onset of study, pretreatment procedures and baseline recording. The changes reported are unlikely to be confounded by dosing or underlying pathology and therefore related to changes in welfare. Furthermore clinical pathology analytes (haematology and clinical chemistry; Study 3) are in line with published values for cynomolgus macaques (Chapter 4), whilst heart rates are lower than those reported in Chapter 4; this is because animals were restrained in a tube for ECG and HDO recording (Chapter 5).

3.4.1 Sensitivity of measures reflecting welfare

Behavioural, physical health and physiological measures in all three studies were found to vary in response to husbandry and scientific procedures performed on macaques. Body weight dropped or stopped increasing in relation to pretreatment procedures. Losses were not severe enough to be in line with recommended humane endpoints (e.g. 10 – 20%). However the greatest loss (mean - 6.9% males; - 6.7% females) observed in animals regrouped prior to pretreatment procedures (Study 2) are similar in magnitude to those reported by Strawn *et al* (1991), following placement in a new social group - a well characterised experimental paradigm used to study cardiovascular disease in cynomolgus macaques. These losses indicated regrouping placed additional stress on animals prior to and during baseline physiological recording, and should be avoided if possible. Body weight did recover and show signs of stabilising by day 1 of dose. However, heart rates and blood pressures in these animals were not elevated compared to macaques in Studies 1 and 3. Nonetheless, heart rates reported in all three studies exceed those reported by Strawn (1991) in freely moving telemetered animals (e.g. baseline: 126 bpm $\pm$ 19; following social regrouping: 164 bpm $\pm$ 24).

Body condition was found to be a useful adjunct to monitoring fluctuations in body weight, and was sensitive to changes in relation to procedural events. Consistent with Clingerman and Summers (2005), body condition was characteristically low in juvenile animals but did show an increasing trend during acclimatisation as animals grew. Body condition dropped during the pretreatment

period following regrouping and baseline recording, and it was slower to recover than body weight before start of dose, indicating that weight loss was likely to be a result of loss of body fat. Thus regrouping in combination with procedural events was having an energetic cost to juvenile animals, diverting resources away from growth.

Increasing severity and incidence of alopecia scores (Honess *et al* 2005) after regrouping may further support the notion that animals were experiencing social stress during pretreatment procedures (Reinhardt *et al* 1986; Honess *et al* 2005). Alopecia in socially housed primates under laboratory conditions most often results from allo-grooming rather than auto grooming (Reinhardt *et al* 1986), and the function of allo-grooming may be in tension reduction following social upheaval or formation and maintenance of social bonds (Schino *et al* 1988; Aureli *et al* 1989; Boccia *et al* 1989; Troisi *et al* 1991). Although it should be noted that female macaques in this study entered the unit with hair loss and may have been experiencing a degree of stress at the suppliers prior to arrival. This may account for the increasing severity of hair loss in these animals throughout acclimatisation even before further regrouping for pretreatment procedures. Nonetheless the link between body weight and body condition loss, and increase in alopecia with social stress cannot be fully confirmed in the absence of behavioural measures, quantifying changes in the interaction of macaques before and after regrouping.

It should be noted that the CASE sponsor tries to ensure animals are housed in their final study groups during acclimatisation to avoid regrouping at start of pretreatment. This is more challenging for toxicologists using macaques than when using other species (e.g. dogs and rodents) due to additional considerations related to transport and supply of purpose-bred macaques (Chapter 5). This study represents exceptional circumstances; it was included in this Chapter to provide additional insights into the sensitivity of welfare measures being tested.

Behavioural responses (e.g. facial expressions, vocalisations, 'handleability', behavioural repertoire and night-time behaviours) of macaques during and following handling and restraint indicate they found husbandry procedures frightening. On a more positive note, fearful responses began to subside significantly over a three-week period (compare week 4 to week 7), with time spent in the

unit. Macaque facial expressions and vocalisations were found to correlate with changes in behaviour in the home pen and with disturbed night-time behaviours, and all three measures co-varied with time in the unit.

The correlating evidence from behavioural observations recorded from macaques in their home pens pre – and post – handling suggests facial expressions and vocalisations given during handling and restraint are reliable indicators of welfare for use in cynomolgus macaques. Indeed Clarke and Mason (1988) and with subsequent publications (e.g. Clarke *et al* 1988; 1994) found fearful facial expressions and harsh calls (e.g. Screech – type vocalisations) were given in response to escalating stressful test situations and correlated with high heart rates (e.g. 230 – 257 bpm) and increased corticosteroid levels.

Increased time spent hugging, embracing and in contact have been reported by other authors in response to transfer to a new facility (e.g. Brent & Veira 2002; Honess *et al* 2004). Furthermore the greater proportions of time spent auto- and allo-grooming observed in week 4 compared to week 7 are likely to reflect a tension-reduction mechanism (Schino *et al* 1988; 1990; Boccia *et al* 1989) in response to the newly formed social groups and novelty (Brent & Veira 2002; Honess *et al* 2004). Indeed these behaviours may help animals to cope with psychological and social stress and highlight the importance of group housing for buffering macaques against stressful laboratory events (Cohen *et al* 1992; Line *et al* 2001; Schapiro *et al* 2001; reviewed in Chapter 4).

In common with other authors (e.g. Kim & Han 2006), macaques were found to follow a distinct circadian pattern of activity in the laboratory, being mainly inactive at night, showing rapid locomotion 30 minutes preceding and following lights on (unreported). However following handling, animals spent more time active and awake and less time apparently asleep, particularly in the hours preceding midnight and this may indicate reduced welfare in response to handling and restraint (Crockett *et al* 1990; 1993; 1995). This finding is inconsistent with Crockett *et al's* (1990; 1993; 1995) findings in macaques following room and cage change. Baseline behaviours observed in the afternoon before handling were more varied than those reported by Crockett *et al* (1990; 1993; 1995), probably as a result of presence of social companions and being housed in large

enriched pens rather than singly housed in small cages. Furthermore, by week 7 increasing amounts of positive behavioural indicators (Section 3.1.1) were seen, such as relaxed locomotion, play, exploration and foraging and feeding.

A review of heart rate and blood pressure and how they vary in response to laboratory events is given in Chapter 5. Heart rates were similar across all three studies and blood pressures were higher in Study 1 compared to Study 2. Despite Refinement of ECG and HDO recording using a tube restraint (Chapter 5) rather than manual restraint (Chapter 4), heart rates and blood pressures are higher than those obtained from freely moving, telemetered animals (Chapter 5).

In Study 2 being 'passive' or 'active' during HDO recording correlated negatively or positively with systolic and diastolic blood pressure. This may be a reflection of hemodynamic principles (Appendix 1.3) rather than directly related to welfare, confirmed by lack of correlation of behavioural responses with either blood pressure or heart rate in Study 1, where animals were mostly passive during recording. However, proportion of Fear3 facial expressions was reciprocally related to being active or passive during handling for ECG in Study 2, thus higher heart rates may be seen in more fearful, 'wriggly' animals. Furthermore, findings of Study 3, which contains the largest cohort of animals, supports the relationship between variation in heart rate and both activity (e.g. hemodynamic principles) and behavioural responses related to welfare (Table 3.3.3 b), specifically alert, relax, Fear3 and Fear2 facial expressions. These compare with Clarke and Mason (1988), and Clarke *et al's* (1988; 1994), findings of positive correlations between fearful facial expressions and high heart rates in response to capture and restraint, exposure to a novel room and physical restraint on a board.

3.4.2 Feasibility of selected measures

Changes in body condition and hair loss were found to reflect changes in macaque welfare in relation to pretreatment events. These measures can easily be incorporated into weekly physical examinations alongside recording of body weights, replacing less quantitative assessments of hair loss and condition. Night-time behavioural measures, however, are not feasible to monitor in primate units without specialist equipment (e.g. cameras and recording equipment). They were

included in this Chapter as way of validation for other behavioural measures of welfare (e.g. during handling and post handling in home cage). To that end they were successful and support the recording of facial expressions and vocalisations given during handling and restraint as reflections of welfare. With appropriate training, the frequency of occurrence (counts) of these types of responses could be periodically recorded on simple check sheets as a litmus test of whether macaques are acclimatising to routine husbandry events. However, some behavioural observations in the home pen may be more problematic (e.g. displacement activities). The occurrence of hugging and embracing is readily identifiable to care staff during numerous daily checks and should be seen as a negative indicator of welfare. Furthermore, simplified check sheets of positive or negative behavioural indicators (e.g. presence of play, feeding and foraging, relaxed locomotion, exploration, hugging, embrace and agitated locomotion) could be used to record behaviour in the home room in a more formalised manner at fortnightly or monthly intervals, and would be no more arduous than undertaking neurobehavioural function assessment tests that often require assessment of behavioural responses in the home cage as well as more specific tests. All indicators of welfare are redundant, however, if they are not reliably recorded and interpreted by care staff. As with unit procedures for monitoring macaque health, comprehensive training should be provided to enable care staff to reliably monitor macaque welfare.

3.4.3 Framework of measures for assessing welfare

Based on the findings of Studies 1 – 3, a framework of measures for assessing the welfare of juvenile cynomolgus macaques is summarised in Table 3.4.1.

Table 3.4.1 A framework of measures for assessing the welfare of juvenile cynomolgus macaques

Measure category	Measure	Recording	Interpretation for welfare		Refer to study findings	Criteria for happiness[b]
			Positive	Negative		
Physical health	Body weight	Weekly	Steadily increasing.	Decreasing or slowed growth when previously gaining weight, unrelated to illness or dosing.	1, 2, 3	(3)
	Body condition [a]	Weekly	Steadily increasing, > 2, not exceeding 3.5.	Decrease, unrelated to illness or dosing; < 3; > 3.5.	2	(3)
	Alopecia	Weekly	1 normal pelage.	Increasing severity >2.	2	(3)
Behaviour	**In-home pen (before handling)**					
	Behavioural repertoire	2h observation, after feeding before final checks.	Broader behavioural repertoire, including: Relaxed locomotion, play, explore, foraging and feeding. Night-time: more time spent asleep before midnight.	Decreased behavioural repertoire, by increasing percentage time spent huddle, embrace, contact, auto & allo-grooming. Decreased time spent asleep before and after midnight, more time spent awake or active.	1	(1, 2,4)
	Response to handling					
	During					
	Facial expression	During handling & restraint	Alert, relax	Fear3,2,1	1, 2, 3	(3)
	Vocalisation		No vocalisations heard	Kra, Wraagh, Khreet Screech	1, 2, 3	(3)
	Handleability		Passive	Active	1, 2, 3	(3)
	Post – handling (2h)					
	Behavioural repertoire	2h observation after handling (compare to Pre-).	Reducing amounts of time spent hugging, embracing, in contact vigilance over 2h post handling.	Decreased behavioural repertoire, by increasing percentage time spent hugging, embracing, in contact, vigilance. No change over observation.	1	(1, 3)
	Post – handling (night-time)					
	Before midnight	Post - handling (compare to Pre-)	Time spent asleep.	Time spent awake or active.	1	(2)
	Midnight onwards	Post - handling (compare to Pre-)	Asleep Awake before lights-on (pre-empting)	Time spent active.	1	(2)
Physiology	Heart rate	Baseline	Lower unrelated to illness or dosing	Higher unrelated to illness or dosing	1, 2, 3	(2, 3)
	Blood pressure	Baseline	Lower unrelated to illness or dosing	Higher unrelated to illness or dosing	1, 3	(3)

[a]Using Clingerman & Summers' (2005) body condition scoring method; [b]Criteria based on Poole's definition of happiness: (1) wide behavioural repertoire; (2) rest in a relaxed manner; (3) confident; (4) not displaying abnormal behaviour.

This framework of measures of welfare will be used to assess the effectiveness of planned Refinements to macaque – care staff interaction in Chapter 5. They will also be used to discuss the effects of changes in housing for macaques used in regulatory toxicology at the CASE sponsor in the next Chapter (Chapter 4). In collaboration with the CASE sponsor this Table will form the basis of a practical and feasible welfare assessment tool to be implemented on site.

4

Refinement of housing and husbandry for cynomolgus macaques (*Macaca fascicularis*) and the effects on biological data

"*SIMIA FASCICULARIS.*
KRA of the Malays.
Frequent in the forests of Sumatra and the Malay islands, where they are met with in large companies. The body is about twenty inches long, and the tail a little more. The back and upper part of the head are of a reddish-brown colour; the tail and sides of the body grey, which becomes still lighter on the inside of the limbs and lower part of the body and face. The face is brown, and covered with short light grey hairs. The cheeks are furnished with tufts of the same colour, much longer than the beard. The eyelids, particularly the upper ones, are white. The eyes are brown, the eyebrows prominent, and the muzzle projecting. The nose is prominent between the eyes, and flat at its point, where the nostrils open obliquely some way above the lip. Cheeks pouched. Ears roundish, obtusely pointed behind. Canines short. Callosites strong. Thumb of the hands short."

Raffles (1821), p 246 - 7.

Description given by Raffles (1821) when surveying the island of Sumatra during his administration of Java. Raffles was the first to give the species its scientific name *fascicularis.*

Abstract

*The last ten years have seen changes in housing for macaques kept in toxicology establishments that are critical for enhanced welfare (e.g. group housing in large, enriched enclosures). These changes reflect an increasing awareness of the social and environmental needs of macaques, yet the hypothesised benefits to scientific outcomes remain unexplored. A data mining study was undertaken to quantify the effects on biological data recorded from cynomolgus macaques (*Macaca fascicularis*) with changes in housing and husbandry spanning an eight year period (2001-8) during which the CASE sponsor transitioned from single to purpose built gang-caging. A secondary aim was to quantify the variation in macaques' data in relation to multiple covariates (e.g. age, sex, acclimatisation time, number of sham doses, and duration of study) that occurred with time. Core battery data were analysed (e.g. baseline body weight change, haematology, clinical chemistry and heart rates, and organ parameters, and from control animals at end-of-life) from 832 macaques included in 28 regulatory toxicology studies. The effects of housing changes on biological data were examined using stepwise, multiple linear regression. The baseline data in this population were broadly in agreement with reference data published for this species. However, the data varied widely (reported standard deviations; ≤10% – ≥50% around population means) depending upon parameter in the core battery. Indeed the degree to which welfare-positive changes in housing affected macaques' biology also varied and housing accounted for a small amount (range 0.1-11%) of variation in baseline data .In some instances the potential welfare benefits of improved housing were confounded by concurrent changes in sham dosing procedures, undertaken in an attempt to habituate animals to this aversive handling and dosing procedure. A large proportion of variation (49 - 99%) in baseline measures could not be explained by the regression model. Indeed this variation is rarely examined across studies, yet this Chapter highlights how many factors impact on baseline levels which may confound interpretation of toxicological data.*

4.1 Introduction

Cynomolgus macaques (*Macaca fascicularis*) used for research and testing in Great Britain (GB) are captive bred but they are not domesticated[1] (Prescott 2010); typically being only one (F1) or two generations (F2) away from wild-caught relatives (Section 1.3). They retain most, if not all, behavioural, physiological and anatomical features that are adaptations to their natural living conditions (Poole 1997; Röder & Timmermans 2002; JWGR 2009). Therefore knowledge of their ecology, biology and behaviour (Section 4.1.1; Table 4.1.1) obtained through field observations and applied studies are fundamental to understanding their needs in captivity. Key features found to be critical for good welfare (Sections 4.1.2 & 3) are increasingly incorporated into legislation and codes of practice (e.g. group housing, environmental enrichment, large enclosures with perches to allow vertical flight; Council of Europe 2007; EU 2010), and the design of new macaque housing for toxicology (Section 4.1.3; Table 4.2.3). Despite the highly regulated nature of toxicology studies (Chapter 1) the effects of changes in laboratory-housing on baseline (pretreatment) biological data derived from macaques have received very little attention in the published scientific literature. Quantifying differences in biological data with welfare-positive changes in housing for toxicology is the main aim of this Chapter (Section 4.1.6).

4.1.1 Wild populations of cynomolgus macaques and their ecology

Wild populations of cynomolgus macaques are found throughout mainland and islands in Southeast Asia and they were introduced on the island of Mauritius, off the coast of East Africa in the Indian Ocean about 400 years ago (Fooden 1991; 1995; Groves 2001; Fooden 2006; see Gumert 2011 for review). They live in a wide variety of habitats (Fooden 2006) ranging from primary, secondary, coastal, mangrove swamp, and riverine forests up to 2000m (Supriatna *et al* 1996; van Schaik *et al* 1996), tolerating humans; they are found utilizing secondary forest, bordering human settlement, where they have access to excess food resources (Sussman & Tattersall 1986; Gumert 2011). Table 4.1.1 summarises their ecology and natural behaviour.

[1] Domestication typically involves selectively breeding animals for their suitability to specific environmental conditions relating to captivity and human use (e.g. factors relating to nutrition, housing, close human contact and climate; McFarland 1981; Hart 1985; RÖder & Timmermans 2002).

Geographic variations result in genetic and phenotypical differences in cynomolgus macaques (e.g. Furuya 1965; Schmidt *et al* 1977; Fooden & Albrecht 1993; Matsubayashi *et al* 1992; Fooden 1995; Butterfield 1997; Migot-Nabias *et al* 1999; Izard & Smith 2000; Groves 2001; Brent & Veira 2002; Leuchte *et al* 2004; Drevon-Gaillot *et al* 2006). Mauritian macaques originated from a small founding population (Kondo *et al* 1993; Lawler *et al* 1995; Krebs *et al* 2005; see Padayatchy 2011 for review) whose origin is unclear, with genetic and morphological information pointing to Sumatran (Tosi *et al* 2002; 2003; Tosi & Coke 2007) or Javan (Sussman & Tattersall 1981) descent. The isolated population of cynomolgus macaques in Mauritius shows a low degree of genetic variation (compared to Asian animals; Leuchte *et al* 2004; Krebs *et al* 2005; Blancher *et al* 2006) and they are relatively disease free (Matsubayashi *et al* 1992); this makes them desirable research models (Harihara *et al* 1988; Tosi *et al* 2003) as both these characteristics are important considerations in the selection of research subjects (Menninger *et al* 2002; Leuche *et al* 2004; Krebs *et al* 2005; Blancher *et al* 2006; Stevison & Kohn 2008). Selecting macaques of greater genetic similarity and higher health status reduces the risk of undesirable variation (e.g. resulting from different genetic X environment interaction; Table 2.2) and confounding factors (e.g. presence of subclinical disease, for example herpes B virus; Table 2.2) during testing.

Table 4.1.1 Ecology and natural behaviour of cynomolgus macaques in the wild

Ecology	Behaviour	Literature
Habitat – primary and secondary forests close to water	Locomotion depends upon habitat In Mauritius, principally terrestrial; in SE Asia, arboreal but able to exploit terrestrial habitats Adapted to climbing and leaping (up to 5m), tails used for balance Good swimmers – may be important for predator avoidance; accessing food Vertical escape routes utilized to avoid predators.	Rodman 1991; Supriatna *et al* 1996; van Schaik *et al* 1996; Sussman *et al* 2011.
Home ranges – 1.25 km^2, highly variable daily path length 150-1900m	Long distances covered when searching for food Feeding (30% of observation time) and moving (23%) are the most common activity observed in cynomolgus macaques in Mauritius.	Wheatley 1980; Sussman & Tattersall 1981.
Diet – diverse, seasonally dependent, primarily frugivorous. Also consume insects, leaves, invertebrates, crabs, frogs, shrimps, clay and bark	Eclectic diet; but selective Cheek pouches enable storage of food during foraging Food is transported away from foraging site for consumption, often to avoid competition from dominant conspecifics.	Wheatley 1980; Sussman & Tattersall 1986; Yeager 1996; Lucas & Corlett 1998; Son 2003; Sussman et *al* 2011.
Activity patterns – primarily diurnal	Activity centred on feeding and foraging in the morning and afternoon; rest during midday Enter sleeping trees (18:00h) and remain until early morning (05:30h).	Gurmaya *et al* 1994 Son 2004.
Sleeping behaviour- roost in groups	Return to same sleeping site each evening Animals sleep huddled together at the edges of branches to avoid ground dwelling predators Branches nearest the top of the tree are chosen, as are trees that border water – predator avoidance involves dropping from trees and swimming away from any potential predators.	Supriatna *et al* 1996; van Schaik *et al* 1996; Sussman *et al* 2011.
Group living (sizes: range <10 to >85 individuals)	Variable group sizes depending on habitat and ecological conditions Stable groups each with a home range Break into subgroups throughout the day Multi-male/multi-female groups Sex ratio: 1 adult male: 3 adult females Females remain in natal groups (philopatry) Males emigrate from their natal group (4-6 years of age) with their peers before they reach sexual maturity. Males may migrate to other groups many times during their life. May exist as solitary males and /or all-male groups.	Angst 1975; de Jong *et al* 1994; van Noordwijk & van Schaik 1999; 2001; Engelhardt *et al* 2004; Sussman *et al* 2011.
Social behaviour- hierarchical	Highly social, hierarchical relationships Organised around matrilines; mothers, daughters & sisters Females - matrilineal dominance hierarchies; males have a strict dominance hierarchy Group cohesion maintained through allogrooming Necessitates complex social communication skills (Chapter 3).	de Jong *et al* 1994; van Noordwijk & van Schaik 1999; 2001; Engelhardt *et al* 2004; Sussman *et al* 2011.

4.1.2 Macaque housing in the laboratory

Accommodation for primates housed in laboratories in Great Britain (GB) has improved significantly over the last decade (Weatherall 2006; JWGR 2009; Table 4.2.3). Gang-caging for macaques is now uniformly installed in contract research organisations (Frost 2004; Finch 2007; Kelly 2007; Archibald *et al* 2009 review the process of change in their respective laboratories). The increased space to accommodate groups also provides opportunities for additional furniture and enrichment items critical for improving the complexity and useable space for macaques (reviewed by Buchanan-Smith *et al* 2004). The provision of an adequate quantity and quality of space for all primates is a requisite for both good welfare (Buchanan-Smith *et al* 2004) and good scientific output (Sherwin 2004; Chapter 2).

Twenty years ago the picture was somewhat different; single housing, in small cages (0.64-0.98m^2 floor area - compared to macaques' physical characteristics) was the norm in four out of five laboratories surveyed by Hubrecht (1994). Caging was constructed of solid walls from aluminium or stainless steel with a mesh or barred front and cage bottom. These fall someway short of current recommendations and legislative requirements (e.g. Home Office 1986; 1989; Council of Europe 2007), and lack many features we now recognise as being important for good welfare (see below). Indeed the reported incidence of abnormal behaviour (e.g. cage-biting, circling, weaving and self-directed behaviour) suggests housing conditions were suboptimal for macaques (Hubrecht 1994; Mason & Latham 2004; Chapter 3).

Similarly, macaques were kept in single (0.8m^2x1.1m), tiered housing at the CASE sponsor laboratory until 2003 (Table 4.2.3). Single housing is both physically and behaviourally restrictive and multi-tier caging creates further challenges. Macaques housed in the different tiers are exposed to differential climatic conditions (e.g. light and humidity), and the nature of the relationship between care staff and macaque may also vary as a result of different physical features between the two levels (Reinhardt & Reinhardt 1999; 2000; Reinhardt 2004). Animals in the lower levels are partially shaded by cages above, experiencing up to a two-fold reduction in light intensity and greater within-cage light-level variation when compared to upper levels (Schapiro *et al* 2000b; Schapiro & Bloomsmith 2001). The

reduced quality and quantity of illumination in the lower tiers makes it more difficult for care staff to visually check animals in those levels (Reasinger & Rogers 2001).

Being kept in small cages inevitably restricts locomotion and physical exercise (Buchanan-Smith *et al* 2004; JWGR 2009); if confined for extended periods of time this may lead to muscle atrophy and decreased joint mobility (Faucheux *et al* 1978; Turnquist 1985). Further to physical restriction, single housing greatly limits the expression of species-typical behaviours (Novak & Suomi 1988; Table 4.1.1) including those which may actually help to reduce the negative impact of laboratory routines (e.g. vertical flight response; Crockett & Wilson 1980) and the effect of social buffering experienced with the presence of conspecifics (Gust *et al* 1994; Kikusui *et al* 2006; Gilbert & Baker 2011).

4.1.3 Changes in housing for macaques used for toxicology

Evolution from single to purpose-built gang-caging reflects scientific knowledge into the negative effects of poor housing and care on behavioural and physiological data (reviewed in Chapter 3 & Section 4.1.4), and a growing industry-wide awareness of primates' social, behavioural and psychological needs (Turner *et al* 2003; Frost 2004; Finch 2007; Archibald *et al* 2009; JWGR 2009). Group-housing in larger enclosures is increasingly a minimum requirement by legislators (e.g. Council of Europe 2007; European Directive 2010/53/EU), and single housing although still permissible for scientific reasons, is under strict provisions and limited to the minimum necessary time (EU 2010).

Historically, the main concern for toxicologists regarding changes to macaque housing was whether studies would be rejected by regulatory bodies (Turner *et al* 2003), owing to the effect housing change may have on type or integrity of data generated and the introduction of additional (undesirable) variation into the experiment aside from that of the test article under study (Kelly 2007). Furthermore, comparison of data generated from macaques in new housing to historical control databases potentially becomes more difficult (Dean 1999; Turner *et al* 2003; Kelly 2007).

In response to these scientific concerns and practical limitations (e.g. large capital investments required for new buildings), change in housing was a staged process; the first step was modifying single caging, connecting single units to enable animals to run together and subsequently converting pens typically used for other large species (e.g. dog pens Table 4.2.3). The baseline biological data from macaques examined in this Chapter span this transition period at the CASE sponsor.

Improved housing has been reported in-house to have positive effects on clinical parameters during toxicity studies (Kelly 2007). Lower incidences and reduced severity of clinical signs with test material were found in macaques housed in groups, in larger pens compared to those receiving the same test article and dose level in single housing. This eventually facilitated testing that particular drug, for the required duration and at a higher maximum tolerated dose, an important aspect in characterising toxicological effects during the safety and efficacy testing of new pharmaceuticals (unpublished data cited by Kelly 2007; Section 1.4.4). In an earlier publication from the host laboratory, macaques group-housed in modified dog pens (Table 4.2.3) were found to have lower blood pressure and heart rates when compared to historic data obtained from macaques housed in single, tiered accommodation (*unpublished* data cited by Dean 1999). These data helped to gain approval from clients for regulatory studies to go ahead (Kelly 2007). Indeed, once new housing has been installed very little concern has been expressed that the gang-caging system could compromise regulatory studies, and no studies carried out in such housing have been rejected by regulatory agencies (JWGR 2009).

Nevertheless, despite the benefits for welfare (Section 4.1.4.), the effect of group housing primates in laboratories on baseline (pretreatment) biological data included in regulatory toxicology studies has not been systematically quantified and published in the scientific literature for macaques, and is the focus of enquiry for this Chapter (Section 4.1.6). This lack of scientific publication is in spite of the conservative nature of toxicological studies, where scientific validation for proposed change is often required, and makes it difficult for facilities to assess changes occurring across industry (Turner *et al* 2003); particularly as many are multi-site, with research operations in countries whose welfare requirements fall significantly below those in Europe.

4.1.4 Cynomolgus macaques' response to the laboratory environment

In order to understand the benefits of the described changes in laboratory housing for macaques we must review their responses to the laboratory environment. Cynomolgus macaques show distinct behavioural and adrenal cortical responses to common laboratory procedures (e.g. confinement in a transport box and room change; Clarke *et al* 1988a, b; Crockett *et al* 1995; 2000; Chapter 3). Moreover they show a greater stress response to the laboratory environment when compared to other commonly used macaque species (Clarke *et al* 1988 a, b; 1994; 1995). For example, Clarke and colleagues (Clarke & Mason 1988; Clarke *et al* 1988 a, b; 1994) found that cynomolgus macaques compared to rhesus (*M. mulatta*) and bonnet (*M. radiata*) macaques had higher corticosteroid levels, heart rates (which declined more slowly) and emitted more distress vocalisations when exposed to novelty and a series of laboratory procedures (e.g. venepuncture, harness fitting, confinement in a transport box and physical restraint on a board). In addition they scored highly for depressed posture, and displayed moderate levels of locomotor activity when placed in a novel room. Furthermore, their mean pretest corticosteroid levels did not decrease across testing conditions, indicating that they habituated more slowly to laboratory conditions than the other two macaque species (Clarke *et al* 1988 a, b). Clarke *et al* (1988 a, b) concluded that cynomolgus macaques were the most distressed of the three groups during their experimental manipulations.

a) Cage sizes and dimensions

Studies that have examined the effects of increasing cage sizes for macaques have yielded conflicting results. Crockett *et al* (1995; 2000) and Bayne and McCully (1989) did not find significant differences in the prevalence of abnormal behaviour in rhesus, cynomolgus or pig-tailed (*M. nemestrina*) macaques housed in larger cages compared with smaller ones. The cage sizes compared, even when at their largest (147% of US Department of Agriculture regulation floor area) were smaller than UK home office guidelines (1989) for macaques weighing 4-6kg (6000cm^2). Moreover they contained no enrichment, either cage furniture or manipulanda, therefore they are unlikely to allow or encourage suitable expression of species-typical behaviour (Buchanan-Smith *et al* 2004). Indeed, small increments of 'dead space' are unlikely to yield significant alterations in animals' behaviour or physiology (Buchanan-

Smith *et al* 2004). Conversely when the complexity and quantity of useable space were significantly increased a reduction in abnormal behaviour and an increase in the amount and complexity of normal behaviours were observed (Draper & Bernstein 1963; Paulk *et al* 1977; Bayne *et al* 1991).

Both horizontal and vertical dimensions of the cage are important for the performance of species-typical locomotor behaviour (Poole 1991). Cynomolgus macaques are primarily arboreal (Crockett & Wilson 1980; van Noordwijk *et al* 1993; Table 4.1.1). It is not surprising, therefore, that in the laboratory Crockett *et al* (2000) found cynomolgus macaques spent more time suspended above floor level than pig-tailed macaques. Their behavioural response to flee vertically when threatened (Crockett & Wilson 1980), becomes more difficult for macaques housed in lower tier cages. Shimoji *et al* (1993) found that cynomolgus macaques in lower cages spent more time on an elevated perch than those in the upper level cages, highlighting, their reluctance to approach the floor (Buchanan-Smith *et al* 2004). Furthermore, cynomolgus macaques exhibit less self-directed stereotypy in vertically enhanced cages compared to standard ones (Watson & Shively 1996). The importance of the vertical escape route may help to reduce the impact of potentially stressful laboratory routines (Reinhardt 1997), as being unable to get away from approach by care staff results in pronounced behavioural and physiological responses (Malinow *et al* 1974; Manuck *et al* 1983; Line *et al* 1989; Bowers *et al* 1998; Boinski *et al* 1999; reviewed in Chapter 5).

b) Single housing

It is now widely recognised that single housing of social primates is detrimental to psychological wellbeing because it does not meet their social and behavioural needs (reviewed in Rennie & Buchanan-Smith 2006a). Cynomolgus macaques naturally live in groups (Table 4.1.1); preventing social interaction added to the effects of small barren caging, greatly limits the expression of species-typical behaviours (Hartner *et al* 2001; Rennie & Buchanan-Smith 2006a). The detrimental effects of social isolation in early rearing environments have been widely studied as models of human development (Section 4.1.5bii). These types of studies found that monkeys raised in isolation are at increased risk of developing severe behavioural pathologies (e.g. self-directed sucking, biting and

aggression, rocking, abnormally passive behaviour and stereotypical behaviour; Harlow & Harlow 1962 a, b; Sacket 1968; Mitchell 1970; Gluck & Sackett 1974) which are indicative of poor psychological well-being (Chapter 3). Such distressing behaviours are also associated with impaired immunological function as characterised by prolonged changes in leukocyte (white blood cells) numbers and measures of lymphocyte function (Laudenslager *et al* 1982; 1990; Coe 1991; 1993; Coe *et al* 1992; Clarke *et al* 1996; Section 4.1.5bii).

The inherent need for social companionship has been recognised as critical for good welfare in most species of primates (Rennie & Buchanan-Smith 2006a), and indeed the presence of a compatible conspecific is the most effective method of enrichment for laboratory-housed primates (Hennessy 1984; Reinhardt 1990; Schapiro *et al* 1996). Indeed, the presence of a companion may act as a social buffer (Kikusui *et al* 2006), reducing stress levels and aiding recovery from aversive experiences (Coe *et al* 1978; Levine *et al* 1978; Mendoza *et al* 1978; Hennessy 1984), thereby helping primates to cope with laboratory events (Rennie & Buchanan-Smith 2006a).

4.1.5 Sources of variation in biological data from macaques

Aside from the effects of changes in housing on macaques' biological data, we should also consider what other variables may alter their biology, thereby potentially impacting scientific outcomes. Over the eight-year time frame (Section 4.2.2) from which historic data could be derived, a number of other variables were found to have changed (Section 4.2.5 & Table 4.2.3). Whilst these were beyond the control of the host laboratory (Section 4.3.2) they may confound our ability to quantify the effects of changes in housing on baseline (pretreatment) data of macaques.

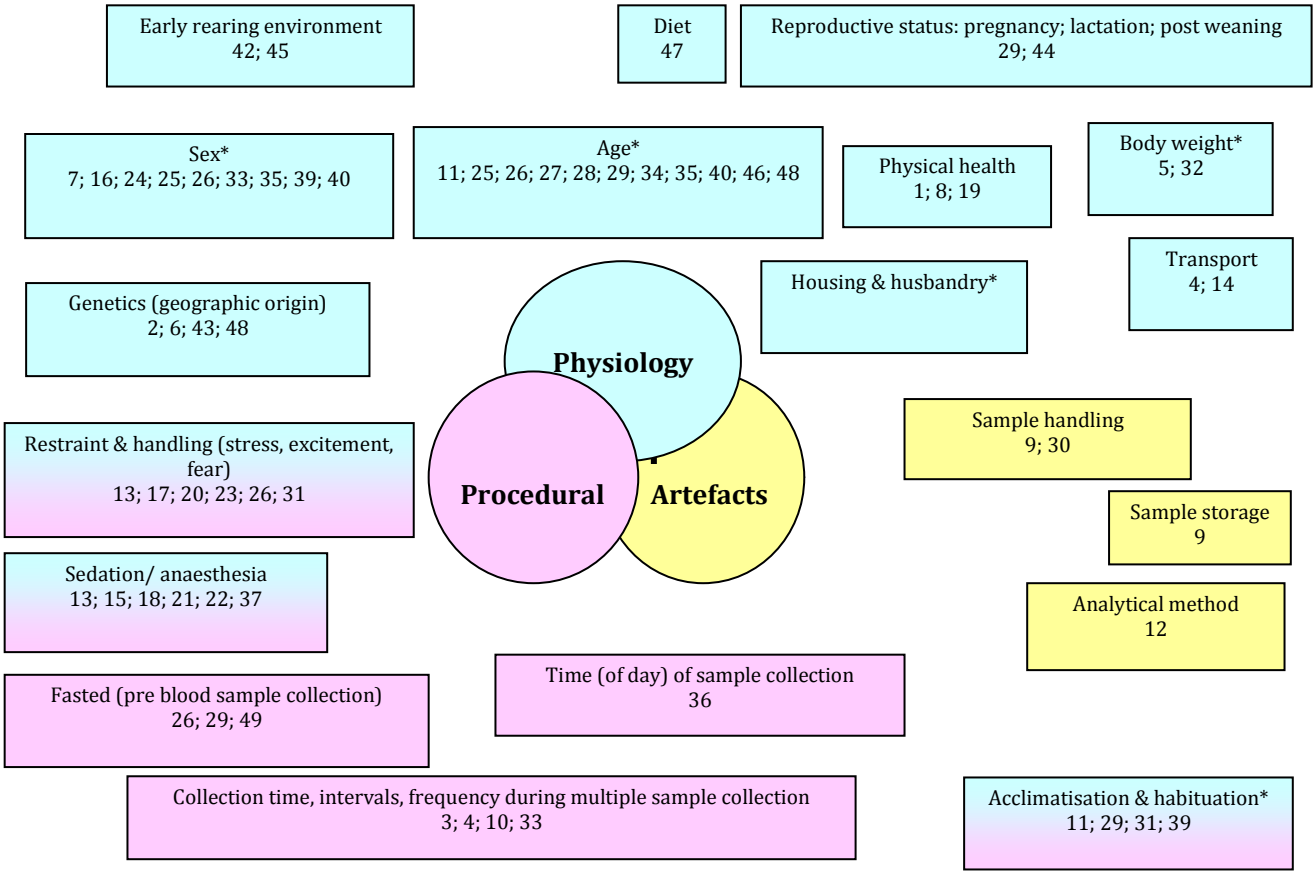
Origin, gender, age, environment and procedures etc. have been found to affect clinical chemistry and haematological (clinical pathology) blood parameters published for cynomolgus macaques (reviewed below). These types and additional (undesirable) sources of variation in data can be loosely categorised as physiological, procedural or artefactual (Hall & Everds 2008; Table 4.1.2) and summarised for macaques in Figure 4.1.1. The main effects of these sources of variation are discussed

below. However, the differences between Asian and Mauritian origin animals are not discussed here, as the CASE sponsor typically uses macaques from Mauritian breeders owing to their enhanced health status and low genetic variation (Section 4.1.1).

Table 4.1.2 Examples of sources of variation in clinical pathology baseline measures (Hall & Everds 2008).

Physiological	Procedural	Artefact
Age Sex Strain Diet Fasted/non fasted Time of sample collection Excitement/fear Stress	Blood collection site & technique Order of sample collection & analysis Anaesthesia Study design factors (e.g. continuous infusion, vehicle, iatrogenic blood loss)	Poor quality sample (e.g. haemolysed or clotted) Improper anticoagulant Too much anticoagulant Improper sample storage

Figure 4.1.1 Sources of variation in blood parameters by category and with examples for macaques.



References (in alphabetical order): 1: Andrade *et al* 2004; 2: Bonfanti *et al* 2009; 3: Capitanio *et al* 1996; 1998; 5: Chen *et al* 2002; 6: Drevon-Gailet *et al* 2006; 7: Giuletti *et al* 1991; 8: Guzman & Radi 2007; 9: Hall 2007; 10: Hall & Everds 2003; 11: Hassimoto *et al* 2004; 12: Hopper & Cray 2007; 13: Ives & Dack 1956; 14: Kim *et al* 2005a; 15: Kim *et al* 2005b; 16: Koga *et al* 2005; 17: Landi & Kissinger 1994; 18: Lee *et al* 2010; 19: Liu *et al* 2008; 20: Loeb 1989; 21: Loomis *et al* 1980; 22: Lugo-Roman *et al* 2009; 23: Mason 1972; 24: Matsumoto *et al* 1980; 25: Matsuzawa & Nagai 1994; 26: Matsuzawa *et al* 1993; 27: Nam *et al* 1998; 28: Pace *et al* 1996; 29: Perretta *et al* 1991; 30: Riley & Cornelius 1989; 31: Ruys *et al* 2004; 32: Schuurman & Smith 2005; 33: Segerstrom & Laudenslager 2009; 34: Sugimoto *et al* 1986; 35: Terao 2005; 36: Terao *et al* 2002; 37: Verlangieri *et al* 1985; 38: Wall *et al* 1985; 39: Wolford *et al* 1986; 40: Xia *et al* 2009; 41: Yoshida 1981; 42: Yoshida *et al* 1987a; 43: Yoshida *et al* 1989; 44: Yoshida *et al* 1992; 45: Yoshida *et al* 1990; 46: Yoshida *et al* 1987b; 47: Yoshida *et al* 1994; 48: Yoshida *et al* 1986a; 49: Zeng *et al* 2010; * Characterised in Chapter 4.

a) Breeding background

(i) Wild caught vs. laboratory bred

Comparison between purpose-bred and wild-caught Mauritian macaques revealed differences in haematology and clinical chemistry, often attributed to differences in health status of animals (Bonfanti *et al* 2009). Eosinophils, large unstained cells (LUC), basophils (B) (Appendix 1.1) were higher in wild-caught than purpose-bred animals, likely reflecting that captured animals undergo greater exposure to infectious agents in their natural habitat (Bonfanti *et al* 2009). In wild-caught animals creatinine

(CREAT), alanine aminotransferase (ALT), and globulins (GLOB) were higher than in purpose-bred animals. Elevated CREAT levels were likely due to differences in muscle mass between the two groups, as creatinine is produced at a constant rate each day by the breakdown of muscle to release energy (Bonfanti *et al* 2009). Elevations of ALT activity may result from release by the liver cells indicative of cellular injury (Hall 2007), hypothesised due to presence of parasites in the liver of captured animals or difference in nutritional habits (Bonfanti *et al* 2009). The commonest causes of elevated serum globulins are inflammatory responses, usually acute-phase, that are likely to have occurred with exposure to infective agents in the habitat of captured animals (Bonfanti *et al* 2009). Of the analytes that were found to differ significantly between the two groups, purpose-bred animals had lower standard deviations in comparison to data from captured animals, and displayed a larger distribution and greater variation around the population mean. The potential for confounds resulting from the reduced health status and greater variation biological parameters in wild-caught animals emphasizes their unsuitability as research subjects for toxicology.

(ii) Early rearing environment

Early experimental work on the effects of rearing infants in total social isolation is extreme in nature, and unsurprisingly produced devastating and irreversible effects on the development, behaviour and physiology of macaque infants (reviewed by Suomi 1997). An umbrella term "isolation syndrome" or "isolate-rearing" was coined to describe the pathological characteristics produced by these paradigms (Parker & Maestripieri 2011). Whilst specific pathogen-free animals may be at risk of developing these devastating pathologies, because in order to minimise risk of mother-infant pathogen transmission, infants are separated at a very early age from the mother and hand-reared, these extreme management practices are not a feature of breeding facilities supplying macaques for toxicology (toxicologists do not require SPF[2] animals *per se*).

[2] SPF: Specific pathogen-free animals; free from defined pathogens or gnotobiotic animals which have no pathogens; they are often produced through early removal of the mother or no contact with the mother (i.e. born by caesarean delivery and hand-reared). Mauritian animals are naturally free of pathogens (e.g. Ebola, B virus, SIV, SRv, STLV-1; Matsubayashi *et al* 1992; Honess *et al* 2010).

We may however see detrimental effects as a result of "accidental" hand-rearing in a peer-rearing paradigm (hand-reared, raised in small groups of same age infants) as an intervention to save the life of a macaque infant in the event of death or rejection by the mother (Honess *et al* 2010). Furthermore, early weaning at 6 months of age was common amongst breeders producing macaques for research and testing. In-light of the detrimental effects of early weaning (see Prescott *et al* 2012)[3], breeders are increasingly delaying this practice until macaque infants are 12 months old, in line with current recommendations for best practice (Scientific Committee on Animal Health and Welfare 2002; Laboratory Animal Science Association and Medical Research Council 2004; Animal Procedures Committee 2006; International Primatological Society 2007; Honess *et al* 2010; Prescott *et al* 2012).

Removal from the natal group and manipulations in the early rearing environment are stressful and result in long-term alterations in the animals' immune system and its regulation (Coe *et al* 1989). More specifically, weaning and removal of the mother is known to have immunosuppressive effects (Coe *et al* 1987), characterised by a decreased lymphocyte and increased neutrophil level (Coe & Scheffler 1989). Even brief maternal separation of socially housed rhesus and pig-tailed macaques produces a shift in immunological function, such as lower mitogen (Laudenslager *et al* 1985; Coe *et al* 1989) and altered cell-mediate responses to a challenge by a specific antigen (Coe *et al* 1989), a shift which may persist up to 9-12 months after reunion (Laudenslager *et al* 1995). Those toxicologists testing new pharmaceuticals that are likely to alter these types of immunological parameters in macaques should be aware of the potential confounding effects of differential rearing histories of macaques when selecting research subjects.

[3] Natural weaning from the mother's milk is usually seen at 14 months of age in macaques (Harvey *et al* 1987); it is a gradual process involving withdrawal of milk and dependence on the mother for care-giving over a period of weeks or months (Lee 1996; Prescott 2001). Offspring remain with their mother beyond weaning up to 24 months of age (Ross 1992). In captive breeding, infants are removed early from the mother and natal group, enforcing abrupt weaning (Honess *et al* 2010).

b) Age- and sex- related differences in clinical pathology analytes

Table 4.1.3 gives age- and sex- related differences reported for clinical pathology analytes in cynomolgus macaques. Only published data from animals in the same age range (juvenile and young adult – Section 4.2.5a) as those included in this study were reviewed, to allow meaningful comparison. Agreement between authors is not always observed; for example CREAT has been found to be higher in males than females (Matsumoto *et al* 1980), higher in females (females>males; Kim *et al* 2005b) or exhibiting no significant difference on the basis of gender (Matsuzawa *et al* 1993). Matsuzawa *et al* (1993) also reported no sex differences in WBC count, aspartate aminotransferase (AST) and ALT after reviewing historic control data from 700 macaques provided by 67 member companies of the Japan Pharmaceutical Manufacturers Association, which disagrees with a number of other published results (Table 4.1.3). However the data were pooled for age, and age ranges were not specified in the text, therefore age*sex-related effects were not determined.

Table 4.1.3 Age & sex related changes in haematology and clinical chemistry analytes for cynomolgus macaques

Age-related				Sex-related			
↑with age	Author	↓with age	Author	Males >females	Author	Females >males	Author
Haematology							
Hb	3;12	**RBC count**	2;3;5;12	**RBC***	1;2;4;5;7;8;9; 10;11;12;13	**WBC**	3;5
WBC*	2;3	**L**	2;5;6	**Hb**	1;2;3;4;6; 8; 9; 10;12	**N**	2;7;9;10
N	2;6	-	-	**Haematocrit (Ht; PCV)**	1;3;4;6;7;9; 10;12	**PLAT**	3;10
-	-	-	-	**WBC***	1;7;9;10	-	-
-	-	-	-	**L**	2;7;9;10	-	-
Clinical chemistry							
ALT ↑ >4y	6	**AST**	5	**ALP**	3;6;7;10	**ALT**	6;7;10
CREAT	5	**ALP**	3;5; 6;12	**AST**	10	**TPROT**	1;12;10
TPROT	5;12	**GGT**	5	-	-	-	-
GLOB	7	-	-	-	-	-	-

1. Matsumoto *et al* 1980; 2. Sugimoto *et al* 1986; 3.Yoshida *et al* 1986a; 4. Giulietti *et al* 1991; 5. Peretta *et al* 1991; 6. Matsuzawa *et al* 1993; 7. Matsuzawa & Nagai 1994; 8. Moore 2000; 9. Andrade *et al* 2004; 10. Kim *et al* 2005b; 11. Schuurman *et al* 2005; 12. Terao 2005; 13. Bonfanti *et al* 2009. * Count. See Table 4.2.5 for abbreviations of haematology and clinical chemistry analytes

c) Effects of transport, acclimatisation and habituation to the new environment on clinical pathology data

Long distance transportation is likely to induce substantial physiological stress in the transported animals (Wolfensohn 1997; Prescott 2001; Honess *et al* 2004; Prescott & Jennings 2004; Hau & Schapiro 2006). Kim *et al* (2005a) have shown that cynomolgus monkeys exhibited an increase in the

neutrophil-to-lymphocyte (N/L) ratio upon arrival at their laboratory. This response normalised a week later, following a similar pattern to serum cortisol, suggesting activation of the HPA axis (Chapter 3) in response to transportation. Similarly, Fernstrom *et al* (2008) measured increased excretion of urinary cortisol in cynomolgus macaques after transportation.

Kim *et al* (2005a) also found a number of other haematological parameters were elevated on arrival: WBC, RBC, Hb, Ht (PCV), mass cell volume (MCV) and platelets (Appendix 1.2). These increases may reflect that animals were dehydrated (elevated RBC, Ht) and fasted (Hb, MCV) as a consequence of transport. Yet these parameters, although lower by day 7 after arrival, continued to decrease across recording sessions to day 35 (last recording), indicating they had not stabilized a month after transportation and housing in the new facility when food and water had been available. Although not explicit during the authors' discussions, the animals displayed less between-animal variation for each haematological variable they recorded on day 35 (last sample) as compared to the first sample (day 0). Yoshida *et al* (1980, below) reported a similar effect after a three-month acclimatisation.

Alongside haematological changes, animals experienced substantial (11.7%) weight loss as compared on arrival to pre-transport body weights (Kim *et al* 2005*a*). Pre-transport body weights were not exceeded until day 21 after arrival in these young adult (3 - 4y) animals, which is a considerable lag-time in response to dehydration and fasting with transport, also indicating that a period of adjustment extends beyond the first seven days after arrival.

Yoshida (1981) found that blood analytes continued to change from arrival over a 3-month adjustment period at a new facility, with RBC, Hb, Ht (PCV), blood urea nitrogen (BUN), glucose (GLU) and albumin (ALB) changing in both absolute values and demonstrating less between-animal variation at three months than when recorded soon after arrival. This has important implications for toxicologists (Chapter 2), who use small numbers (2 - 6) of animals per treatment group, with animals acting as their own controls and statistical analysis being performed on group mean values for each parameter to identify and understand mechanisms of potential toxicity (Section 1.4.4).

Capitanio *et al* (2006) recently reviewed the effects of housing changes on a variety of physiological parameters for commonly used Old World monkey species in the laboratory. They too concluded that it may take up to 90 days for macaques to adapt to new housing conditions, depending on the level of change they experienced. Hassimoto *et al* (2004) reports a more complicated picture in their extended study following rhesus monkeys through 6 month's acclimatisation and habituation to procedures, finding that each analyte in the core battery took different lengths of time to stabilize and varied according to gender. For example, heart rate was higher in males than females, decreased across recording sessions, significantly by month 3 for females, month 4 for males, but not stabilizing until 5 months in males. Haematological data were more complicated; WBC and platelets decreased over time for both sexes, stabilizing at different rates over the 6-month recording period, whereas RBC, Hb and Ht (PCV) fluctuate each month, in different directions for each sex. Consistent changes in clinical chemistry parameters included AST and ALT, which did not significantly change over 6 months, whereas ALP and CREAT decreased, both of which were thought to be related to growth in monkeys.

These studies indicate that the physiological changes that occur with transportation and relocation persist for some time after arrival at a new facility, and in addition there may be greater between-animal variation if studies are conducted before these blood analytes have stabilized, which could conceivably confound experimental outcomes (Obernier & Baldwin 2006; Chapter 2) for toxicologists as they employ a repeated-measures experimental design (Section 1.5.4).

d) Restraint

The effects of restraint on ECG and blood pressure parameters have been reviewed in Chapter 5 and are not included in the background review of this Chapter. The endocrine response to restraint is reviewed in more detail in Chapter 3. The effects of chemical restraint and biological parameters are not reviewed as it is not common practice by the CASE sponsor to obtain study data from sedated animals.

Physical restraint has been shown to be a powerful physiological and psychosocial stressor for monkeys. It activates the HPA axis (Morrow-Tesch *et al* 1993; Chapters 3 & 5) and results in catecholamine and corticosteroid release, which leads to pronounced and far-reaching effects on laboratory data (Hall 2007), including metabolic (Manning *et al* 1969), physiological (Bush *et al* 1977; Line *et al* 1987) and haematological and clinical chemistry parameters (Yasuda *et al* 1988; Landi *et al* 1990).

Ives and Dack (1956) first reported an "alarm reaction" in rhesus macaques manually restrained (whilst conscious) in a restraining box for an experimental protocol and to facilitate safe venepuncture (from untrained animals). Restraint resulted in elevated WBC counts and significant alterations in differentials, namely a decreased neutrophil and elevated lymphocyte and monocyte counts. The effect was most pronounced upon first restraint, and decreased over successive months on study, as animals showed habituation to handling and restraint (Ives & Dack 1956).

Morrow-Tesch *et al* (1993) also found an increased WBC count following brief ketamine anaesthesia to facilitate handling and placement of rhesus macaques into a restraint chair for up to 3 hours. This later work contradicts the effects of restraint on leukocyte differentials found by Ives and Dack (1956), with increased neutrophil, decreased lymphocyte and monocyte percentages recorded. The effects on WBC and differentials got larger with increasing lengths of time in the restraint chair. There was a significant negative correlation between the N/L ratio and duration of passivity. The elevated cortisol and B-endorphin levels also indicated that immune changes were mediated by the stress-response. Similarly, Capitanio *et al* (1998) found increased neutrohpils and decrease lymphocytes in rhesus macaques in response to two-hour chair restraint.

Comparison of restraint methods and their effects on ALT and AST, important indicators of liver function and damage to liver, cardiac and skeletal muscle (Hall 2007), revealed that these too are significantly elevated by restraint in cynomolgus macaques (Landi & Kissinger 1994). Restraint methods compared included a Perspex box, chair, board or manual restraint by technician. Repeated

venepuncture and subsequent analysis found that all methods of restraint resulted in elevations of ALT and AST over time (up to 2 hours) compared to baseline. Elevations in AST were seen earlier and were greater than those for ALT. Board restraint produced the highest AST and ALT levels recorded, and chair restraint had the least demonstrable effect; this was thought to be because chair restraint gave the animals the greatest freedom of movement. The level of AST and ALT continued to be higher than that of baseline at 72 hours after the first sample, but were close to baseline levels by 168 hours after the first sample was taken. The greatest between-animal variations were seen when maximum concentrations of AST and ALT were recorded 6-24h after the first sample. The variations of ALT and AST levels induced by restraint may increase unwanted data variability and confound drug-related effects occurring in the liver and muscle tissue.

e) Procedural effects

Capitanio *et al* (1996) explored the effects of common laboratory disturbances associated with blood collection on the variability of haematological data recorded from rhesus macaques. Pen location, and amount of visual and auditory disturbance macaques experienced when blood was collected sequentially from animals in a single room were found to be significant contributors to variation in counts of circulating leukocyte (WBC) sub-populations. Higher cortisol and lower lymphocyte counts were recorded for animals in the bottom row of tiered housing, whose visual access was restricted, whereas macaques located in higher pens that were able to see other macaques experiencing venepuncture had 28% higher lymphocyte counts. In a second experiment eliminating the visual variable, disturbance time, time from auditory disturbance of technical staff preparing for venepuncture to blood sample, was found to exert a significant effect on variation of haematological parameters; a 60% increase in the number of circulating immune cells was recorded within 9 minutes. Disturbance-induced changes were found in CD8+ T lymphocytes, CD4/CD8 ratio and neutrophils, although these were secondary to ACTH and cortisol release as these were found not to be related to disturbance time. CD8+ T lymphocytes were more than 50% higher in animals sampled 6-9 minutes after onset of disturbance than those within 3 minutes. CD4/CD8 ratio was higher between 6-9 minutes after onset of disturbance and neutrophil counts were higher after 12 minutes of disturbance.

Disturbance time was found to be a significant contributor to variance in leukocyte subset counts, with each of the immune cells affected at different time points. Such variation may lead to erroneous clinical decisions and inaccurate research results (Capitanio *et al* 1996).

4.1.6 Aims of Chapter 4

Group-housing in large, enriched pens has been found to be positive for primate welfare when housed in the laboratory in comparison to small, single cages (Section 4.1.2). However, quantification of the effects of welfare-positive changes in housing for cynomolgus macaques on their baseline data recorded for regulatory toxicology has not been systematically examined in the scientific literature. This was the principal aim of this Chapter. Secondary, but equally important for interpretation of effects of housing was to quantify variation in macaques' baseline data in relation to multiple covariates (age, sex, acclimatisation time, number of sham doses, and duration of study) that occurred with time.

4.2 Methods

4.2.1 Biological variables included for analysis in a core battery of measures

This chapter examines the effects of housing and their associated husbandry changes (Table 4.2.3) on the biological data recorded as part of a core battery of measures (Section 1.5.3 & Figure 1.2) from macaques of Mauritian origin in regulatory toxicology (Section 1.4.4 & Figure 1.3). Included for analysis were body weight changes (BWC), heart rate (HR), selected haematology (H) and clinical chemistry (CC) analytes obtained from all animals' pretreatment (baseline) and selected organ:body weight ratios (O:BW) for control animals at end-of-life (Table 4.2.4 & 6).

Seventeen haematology and clinical chemistry analytes (Table 4.2.5) from the core battery were selected because they were considered biologically important for assessment of welfare (Chapter 3) and for determining the biological findings of toxicity and safety studies (Weingand *et al* 1996; Appendix 1.1). They are critical components of the core battery of blood parameters recommended by Hall (2007) allowing characterisation of effects on the immune system, oxygen carrying capacity of the

blood, hepatic and renal function (Appendix 1.1). Blood glucose, total cholesterol and triglycerides were not included for analysis because it was not possible to quantify the variation in serum levels with respect to changes in housing as they are dependent upon both dietary intake, where different diet brands were given to macaques during the intervening study period 2001-2008, and endogenous factors such as synthesis by the liver and tissue uptake, which may vary with stress in relation to housing and husbandry conditions (Hall 2007).

Organs included have specific metabolic, immune or endocrine functions (e.g. adrenals, pituitary, thyroid, liver, spleen and kidneys). Organs were excluded if they were likely to show maturity-related tissue variability owing to their role in production and modulation of sex hormones or reproductive activity (e.g. testes, prostrate, ovaries and uterus). Organ:body weight ratios were derived from end-of-life body weights and weight of individual organs at *post mortem* as recommended by Sellers *et al* (2007) to eliminate variations due to body weight differences. Organ:body weight ratios were not included from two studies (13 & 14) in housing condition B (Table 4.2.2) as animals were dosed more than once a day, and the effects of associated capture and handling for more frequent dosing would introduce additional variation into the regression model. The biological variables are termed 'outcome variables' (Field 2005) for the purposes of analysis by multiple regression (Section 4.2.8).

4.2.2 Mining historic data

Data were mined from historic study reports and study data archived by the CASE sponsor during 2001 - 2008 according to the criteria given in Table 4.2.1 – these were considered typical characteristics of regulatory toxicology studies for the host laboratory. Twenty-eight general (regulatory) toxicology studies were selected and provided data for 420 male and female cynomolgus macaques (N = 840). During this time period toxicology studies were conducted on macaques housed in three different conditions (Table 4.2.3); modified single (A) and modified dog (B) housing ran concurrently until March 2003 when they were replaced by purpose built gang-housing (C; Section 4.1.3). Studies undertaken 12 months after change in housing (March 2003 - March 2004) were excluded from analysis. Demographic data are displayed in Table 4.3.1. Studies were assigned a code (1-14: pre March

2003 modified single and dog; 15-24 post March 2004 purpose built gang) for sponsor anonymity (Table 4.2.2).

Table 4.2.1 Criteria for mining data

	General Factors	Study-related factors	Laboratory factors
Actively selected	**Species** - cynomolgus macaques. **Origin** – Mauritius **Supplier** – same Mauritian breeder. **Sex** – single-sex studies were excluded. Each study contained equal numbers of male and female macaques. **Restraint** – manual restraint by technician with the macaque held in dorsal recumbancy.	**General toxicology study –** data from animals included in studies for regulatory toxicology. **Route of administration** – the same route of administration of test article - oral gavage (OG), the most common dose delivery route. Animals were sham dosed prior to day 1 of dose during week-1 coinciding with the pretreatment (baseline) recording. **Baseline (pretreatment)** – data were included from all animals prior to onset of dosing with test article. **End-of-life** – organ:body weight ratios were included from control animals only. These animals were sham dosed throughout study and did not receive the test article. Only control animals that received once daily dosing were included.	None – see below
Recorded not controlled for	**Age** – studies contained juvenile and young adult animals (Section 4.2.5a)	**Duration of acclimatisation**– animals were housed in study rooms prior to onset of dosing to acclimatise to local conditions. The length of acclimatisation varied with study (Table 4.3.1). **Blood volume** – varied with study as other blood analysis in addition to the core battery of clinical pathology measures were sometimes undertaken. **End-of-life** - duration of study varied and this was included as a predictor variable (Section 4.2.6) for organ:body weight ratios only.	**Method** – same general analytical and data capture methods were used for determination of outcome variables (Table 4.2.4 & 5)

Table 4.2.2 Demographic data for 28 studies selected for analysis across all housing conditions

H		C	Yr	Dos	Dur (w)	N (M:F)	Nc (M:F)	Data included for analysis						
								Acc (w)	Age (w)	BWC (g)	Pretreatment			End
											HR (bpm)	H	CC	O:BW (%)
Pre-2003 modified single & dog	A	1	2001	1	2	14 (7:7)	2 (1:1)	*	*	•	x	•	•	•
	B	2	2002	1	4	24 (12:12)	6 (3:3)	•	•	•	•	•	•	•
	B	3	2002	1	4	24 (12:12)	6 (3:3)	*	*	•	•	•	•	•
	B	4	2002	1	4	24 (12:12)	6 (3:3)	•	•	•	•	•	•	•
	A	5	2001	1	2	42 (21:21)	6 (3:3)	*	*	•	•	•	•	•
	B	6	2001	1	13	40 (20:20)	12 (6:6)	*	*	•	•	•	•	•
	B	7	2001	1	13	32 (16:16)	8 (4:4)	•	•	•	•	•	•	•
	B	8	2003	1	13	40 (20:20)	8 (4:4)	•	•	•	•	•	•	•
	B	9	2002	1	4	30 (15:15)	8 (4:4)	•	•	•	•	•	•	•
	A	10	2002	1	4	32 (16:16)	10 (5:5)	•	•	•	•	•	•	•
	A	11	2002	1	13	24 (12:12)	6 (3:3)	•	•	•	•	•	•	•
	B	12	2003	1	2	24 (12:12)	6 (3:3)	•	•	•	•	•	•	•
	B	13	2002	3	13	40 (20:20)	12 (6:6)	•	•	•	•	•	•	•
	B	14	2003	2	4	32 (16:16)	8 (4:4)	•	•	•	•	•	•	•
Post 2004 purpose-built gang	C	15	2005	1	13	16 (8:8)	8 (4:4)	•	•	•	•	•	•	•
	C	16	2005	1	2	24 (12:12)	6 (3:3)	•	•	•	•	•	•	•
	C	17	2005	1	13	32 (16:16)	8 (4:4)	•	•	•	•	•	•	•
	C	18	2005	1	4	32 (16:16)	10 (5:5)	•	•	•	•	•	•	•
	C	19	2004	1	13	40 (20:20)	8 (4:4)	•	•	•	•	•	•	•
	C	20	2006	1	4	32 (16:16)	10 (5:5)	•	•	•	•	•	•	•
	C	21	2004	1	13	42 (21:21)	6 (3:3)	•	•	•	•	•	•	•
	C	22	2007	1	4	24 (12:12)	6 (3:3)	•	•	•	•	•	•	•
	C	23	2007	1	13	32 (16:16)	8 (4:4)	•	•	•	•	•	•	•
	C	24	2005	1	13	40 (20:20)	10 (5:5)	•	•	•	•	•	•	•
	C	25	2005	1	4	24 (12:12)	6 (3:3)	•	•	•	•	•	•	•
	C	26	2006	1	4	24 (12:12)	6 (3:3)	•	•	•	•	•	•	•
	C	27	2007	1	4	32 (16:16)	10 (5:5)	•	•	•	•	•	•	•
	C	28	2008	1	4	24 (12:12)	10 (5:5)	•	•	•	•	•	•	•
T		**28**				**840**	**216**							

Data obtained: • Data from database and study report; * Estimate from study report; **H** Housing condition: A Modified single; B Modified dog; C Purpose built gang; **C** Assigned code for each study; **Yr** Year study started; **Dos** Frequency of daily dosing with test article on study; **Dur** Duration of study (weeks) – Day 1 of dose to end-of-life; **N** Total number of animals included in pretreatment (baseline) recording; **M** Male; **F** Female; **Nc** Total number of control animals; **Acc** Length of acclimatisation time (weeks); time from arrival to day 1 of dose; **Age** Age of animal (weeks) at start of study; **BWC** Body weight change (g) week-2 to day 1 of dose; Pretreatment data (all animals): **HR** Heart rate (beats per minute); **H** Haematology; **CC** Clinical chemistry; **End** *Post mortem* data for control animals: **O:BW** organ/body weight ratio (%).

4.2.3 Housing and husbandry

Animals were housed and cared for in accordance with the Animals (Scientific Procedures) Act (1986) and associated Codes of Practice (Home Office 1986; 1989). Animal rooms were maintained at temperature range 18-24^{o}C, relative humidity 30-80%, air ventilation minimum 10 air changes per hour, and 12 hour light/dark cycle. Water was available *ad libitum* and supplemented daily with a diluted mixed fruit drink. Macaques were fed a standard pelleted commercial primate diet supplemented with a range of food stuffs on alternate days (e.g. fresh fruit, forage mix, dried fruit and nuts). Food was scattered on the floor in housing conditions B and C and placed in troughs in housing A. Cage sizes and characteristics are outlined in Table 4.2.3; cages were furnished with perches and enriched with toys, and in housing conditions B and C wood shavings were placed on the floor. Modified single housing (A) had grid floors, with a removable tray underneath for collection of animal waste, and pens were arranged in two-level stacked tiers. Animals were acclimatised to local housing conditions and were examined for acceptability onto study by a veterinary surgeon; they were therefore considered clinically normal at the time of pretreatment procedures.

Table 4.2.3 Comparison of housing of cynomolgus macaques 2001–2008 at the CASE sponsor

	Description	Qualitative features
(A) Modified single	**Pen dimension -** Single pen designed to accommodate one animal, 1.1m high; 0.8m^2 floor area. **Pen construction** - Stainless steel, with bars at the front, grid floor, solid back, sides and partitions. Stainless steel bars for perching off the grid floor, running along the depth of the pen on the right hand wall. Rows of six pens, two tier arrangement of pens either side of the room with a central walk way for care staff. **Social contact** - Single-sex, group housed during acclimatisation period by removing partitions between pens and running animals together. Separated into single housing during baseline recording (partitions replaced) and dosing during the day, group housed (partitions removed; dose groups only run together) at end of working day. **Food presentation** - Feed troughs on the front of each pen. **Capture** - Hand-caught by technician standing at the front of the pen. **Enrichment** – Limited opportunities.	Small living space, vertical height and floor area, limits opportunity for normal locomotive behaviour; walking, climbing, swinging especially when pens separated. Cannot perch in cage with tail freely suspended. Unable to flee vertically when frightened or to get away from care staff. Reduced light intensity to the lower tier of pens, owing to stainless steel collection tray for the upper row. Potentially noisy, especially if animals display cage shaking, cage banging (Chapter 3) and likely during normal husbandry operations e.g. feeding, cleaning of waste trays, capture etc. Social, tactile contact prevented and visual contact reduced when pens separated during working day whilst on study; little opportunity to display normal coping behaviour to potentially stressful events post - capture, handling, restraint, dosing and recording (Table 4.1.1; Chapters 3 & 5). Length and width of perching bars limit number of macaques that can sit comfortably together and stretch out during allo-grooming. No foraging opportunities More difficult for care staff to socialise with animals in lower tier. Potential for conflict with care staff during capture; technicians have to be 'face-on' and bring macaques out directly into their face/body during capture (Chapter 5).
(B) Modified dog	**Pen dimension** – Single pen can accommodate up to 6 animals, 2m high; 2.25m^2 floor area*. **Pen construction** – Stainless steel bars at front and side of pen with additional grids to prevent animal escape on roof, front, and sides (adapted from dog pens). Solid floor, covered in shavings. Two solid suspended benches covered in rubber matting running the length of the back wall. One sited near to the top of the pen with sufficient height to allow macaques to stand full height without touching their heads on the roof of the pen. One suspended halfway between the floor and the top bench. Single row of pens four each side of a central aisle. **Social contact** - Single-sex group housed throughout acclimatisation period, baseline recording and dosing. Up to four animals when on study. **Food presentation** – Food scattered throughout the pen, on the floor and benches. **Capture** – Hand-caught into transport boxes by technician. Technician enters pen. **Enrichment** – Swings and toys suspended from the ceiling, of the pen, additional manipulandi and foraging opportunities.	Larger living space, animals permanently group housed. Pen construction allowed for more normal locomotive behaviour and additional enrichment items for perching swinging, and foraging. Greater opportunity to display coping behaviour and control over potential stressors e.g. vertical flight reaction, sit off the floor and away from technicians, social contact etc. Macaques can sit, huddle, sleep and stretch out during allo-grooming comfortably altogether on the suspended shelves. Potentially less noisy than housing (A) owing to stainless steel mesh rather than solid partitions and less discrepancy in light intensity levels than tiered system. May still be potential for conflict with care staff during capture as they enter the home pen and hand capture macaques.

(C) Purpose built	**Large animal holding unit** – animals received from supplier into this unit and held from arrival until transfer into main study unit for acclimatisation to local conditions. Pen design flexible and can accommodate 30 plus animals held in single sex groups. Large stainless steel mesh sides and top, sold floor, numerous perches and swings etc., varied with each group. **Study unit (full details given in Chapter 3) -** **Pen dimension** – Single pen can accommodate up to 6 animals, 2.4m high; 2.25m^2 floor area*. **Pen construction** – Stainless steel widely spaced bars up the front and top of the pen. Solid floor and solid walls with removable partitions. Floor covered with shavings. Single row of pens, four each side of a central aisle. **Shelving and veranda** – 3 suspended wooden shelves 0.75m^2 floor area plus wooden veranda or viewing platform above the door. **Social contact** - Single-sex group housed throughout acclimatisation period, baseline recording and dosing. Up to four animals when on study. **Food presentation** – Food scattered throughout the pen, on the floor and benches. **Capture** – Box trained, macaques trained to run into opaque Perspex transport boxes, by a process of habituation and desensitisation (with some negative reinforcement). Transport box placed at the front of the 'hatch' by technician. Technician sometimes enters pen to encourage animals to run into transport box. **Enrichment** – Swings and toys suspended from the ceiling, of the pen, additional manipulandi and foraging opportunities.	Same qualitative features as housing (B) but improved with additional perching space for greater choice and viewing platform (veranda) at the front of the pen to 'spy' on care staff and neighbouring pens (Chapter 5). Wider bars allow protected contact with care staff without entering macaques' home pen (Chapter 5). Potentially least noisy construction materials in comparison to other housing (A & B). Reduced conflict with care staff during capture as animals are trained to run into a transport box, placed on the outside of the pen.

* Under the Home Office Code of Practice for the housing of animals used in scientific procedures (home Office 1989).

4.2.4 Sampling and recording techniques in-life and *post mortem*

Tables 4.2.4 & 5 give the recording and analytical methods for biological (outcome) variables by the CASE sponsor.

Table 4.2.4 Sampling and analysis techniques for in-life and end-of-life data collection

Biological (outcome) variable	Recording & analysis method	Population included for analysis
Haematology & clinical chemistry (H&CC)	**Venepuncture method -** single-use disposable hypodermic fixed needle attached to disposable plastic syringe sized according to total volume of blood withdrawn (study dependent). **Venepuncture site** – femoral vein or artery. **Fasted** – overnight prior to venepuncture. **Analysis** - automatic and manual blood smear (Table 4.2.5). **Analysers and reagents -** three different analysers and reagents were used (Table 4.2.5), quality assurance measures in place ensure results are within comparable limits of determination.	All animals included in pretreatment (baseline) procedures[1]
Heart rate (HR)	**ECG recording -** 30-60 seconds of trace were recorded from conscious restrained animals, using Microsmart MC Dogs ECG machine from Marquette Hellige Medical Systems on paper set at a continuous speed (50mm/Sec). Heart rate was derived from Lead II (Appendix 3.2). **HR calculation -** trace was visually appraised by the study director and mean heart rate (bpm) derived from 11 R-R peaks (10 heart beats) (Appendix 3.3.2).	
Body weight (BW)	**Body weight recording -** animals were weighed weekly from arrival and throughout study using automatic digital weigh-scales. Body weight (g) was recorded manually by technician or automatically using an in-house electronic data capture system. Body weight change – calculated: Week -2 to week -1 to day 1 of dose at start of study (Figure 1.3) were included for analysis. Animals were weighed before the dose of test article was given on day 1 of study.	
Organ:Body weight ratio (O:BW)	**Organs** - excised at end-of-life and weighed in grams (g) immediately after removal. The ratio of organ weight to end-of-life body weight (O:BW) was calculated (%).	Control animals (sham dosed throughout study)[1]

[1]Where data were collected (Table 4.2.7)

Table 4.2.5 Haematology and clinical chemistry analytical methods

Analyte		Unit	Methods	Analysers and reagents
Haematology				
Haemoglobin concentration	Hb	g/dL	Cyanmethemoglobin spectrophotometry	Analyser: Bayer ADVIA; Reagent kit: Bayer Diagnostics Ltd
Red blood cell count	RBC	Mil/cmm	Flow cytometry	
Packed cell volume	PCV	%	Calculation from MCV	
Platelets	PLAT	1000/cm 10^9/L	Flow cytometry	
White blood cell count	WBC	10^9/L	Flow cytometry Manual: Blood smear Automated: Perox optical with chemical reaction	
Neutrophils (absolute)	N (abs)	10^9/L		
Lymphocytes (absolute)	L (abs)	10^9/L		
Monocytes (absolute)	M (abs)	10^9/L		
Clinical chemistry				
Aspartate aminotransferase	AST	IU/L	UV rate	**Method A** - Analyser: Hitachi 917/ILAB 900 Reagent kit: Boehringer Mannheim UK LTD **Method B** - Analyser: HITCAHI 917/ILAB 900 **Method C**- Analyser: Roche P Module
Alanine aminotransferase	ALT	IU/L	UV Rate	
Gamma glutamyltransferase	GGT	IU/L	GGCN	
Alkaline phosphatase	ALP	IU/L	P-NPP	
Urea	UREA	mmol/L	UV rate	
Total bilirubin	T BILI	mmol/L	Colorimetric D	
Creatinine	CREAT	mmol/L	Colorimetric	
Total protein	T PROT	g/L	Biuret	
Globulin	GLOB	g/L	Calculation: GLOB= T PROT – Albumin (not reported)	

Clinical chemistry analysers and reagents: 3 different analysers were used by the CASE sponsor during 2001-2008; these were not included as 'predictor' variables in this study, as the CASE sponsor ensured results from different analysers were within comparable limits of determination.

4.2.5 Additional sources of variation

During the course of data mining, owing to the wide time frame from which the study populations have been derived, a number of other sources of variation that are likely to confound the effects of housing changes were identified (Table 4.2.6). For example, macaques in the purpose-built gang-caging were older and acclimatised for less time than other housing conditions. Sham dosing was only performed once in the modified single housing condition, and was increasingly performed by the time of purpose-built housing. These variables are termed 'predictor variables' (Field 2005) and had to be characterised for inclusion into the regression model for analysis (Table 4.2.6).

4.2.5a Age groups

Age in weeks at start of study could not be determined for all animals in studies 1, 3, 6 and 7 (Table 4.2.2), hence an estimate was taken from the original study report and all animals were divided into age bands based upon maturity using the same criteria as Andrade *et al* (2004)[4]: juvenile 55 – 129w; young adult: 130 - 251w. This allowed construction of reference intervals (Section 4.2.7) that were biologically meaningful.

4.2.6 Data analysis

Data screening and analysis were undertaken in stepwise manner; data were screened for normality, reference intervals were calculated for the entire population, data were transformed where necessary and the effects of age and sex were examined separately. Finally the combined effects of all the predictor variables (Table 4.2.6) were examined using a multivariate regression model; this allowed quantification of changes in housing alongside other variables on macaques' biological data. Analysis of Covariance with contrasts were performed using predictor variables found to be exerting a significant effect on variability of the data to allow comparison between housing conditions.

[4] Animals aged 60 - 77w were classified as juveniles and not infants post weaned as in Andrade *et al* (2004), as they were no longer with their mothers.

Table 4.2.6 Defining known predictor variables and outcome variables for multiple regression

Outcome (biological) variable				Predictor (identified sources of variation) variable			
Variable	**Unit**	**Description**	**Regression model**	**Variable**	**Unit**	**Description**	**Regression model**
H Hb RBC PCV PLAT WBC N L M	 g/dL mil/cmm % 1000/cmm 10^9/L 10^9/L 10^9/L 10^9/L			Housing	-	Categorical	A Modified single (1) B Modified dog (2) C Purpose built gang (3) Category Converted into dummy data for regression, C kept constant (post 2004 housing)
CC AST ALT GgT ALP UREA TBILI CREAT TPROT GLOB	 IU/L IU/L IU/L IU/L mmol/L mmol/L mmol/L g/L g/L	Continuous	Entered as continuous data	Sex SD Maturity Age Acclim	- - - W W	Discrete Categorical Discrete Continuous Continuous	Male (0) Female (1) Count Juvenile (2) Young Adult (3) Entered as continuous data
HR	BPM						
BWC	g						
O:B Adrenals Kidneys Spleen Liver Pituitary Thyroid	%	Continuous, derived (Table 4.2.4)	Entered as continuous data	Duration	W	Continuous	Entered as continuous data

W: weeks; SD: Number of sham doses

4.2.6a Data screening

Raw data were entered into Microsoft excel (2007) for initial screening and then transferred for analysis to the Statistical Package for the Social Sciences (SPSS) v. 18 (2010). The data set was incomplete either as a result of spoiled samples, or individual analytes were not recorded for that particular study.

Many biological analytes are not normally distributed around a central mean (Harvey *et al* 2008). Histograms for each analyte were visually inspected and examined for normality using Kolmogorov-Smirnov (K-S), and additional Z scores for skewness and kurtosis were calculated (Field 2005):

$$\text{Z skewness score} = \frac{S-0}{SE_{skewness}} \qquad \text{Z kurtosis score} = \frac{K-0}{SE_{kurtosis}}$$

The values of S (skewness) and K (kurtosis) and their respective standard errors are produced by SPSS. Absolute values of Z, above 3.29 were considered significant and data deviate from normal.

However, owing to the large data set >200 samples (N=840), Field (2005) cautions that even small deviations from normal become significant when using K-S and Z scores. The problem lies in determining whether this is enough to bias any parametric statistical procedures applied to the data. Field (2005) recommends examining histograms and normality plots to aid decisions regarding acceptability for parametric analysis. Any data found not distributed normally were transformed using log $Base_{10}$ to enable parametric analysis. Normality was confirmed by replotting and visually examining the transformed data, and performing K-S analysis, and calculating Z scores for skewness and kurtosis. Predictor variables do not have to be normally distributed for inclusion into multiple regression models (Field 2005). Descriptive statistics were performed to characterise predictor variables (Table 4.2.6) before analysis.

4.2.7 Defining the characteristics of the overall population

The host facility maintains a database of historic control[5] data compiled from studies undertaken during June 2000 – March 2007. The data are derived from Mauritian animals, and pooled for housing and supplier, and stratified by age in weeks (in 20 week blocks). Making the historic control data unsuitable for comparison to the selected population. Equally undesirable was to use published normative values (Appendix 1.2) as a benchmark for quantifying change in relation to housing, as they are often derived from macaques of Indo-Chinese origin rather than Mauritian, and from too few animals, below the number recommended, minimum 100 – 120 individuals, by the National Committee for Laboratory Standards (1992) for constructing reference intervals (Lumsden 2000). Therefore data

[5] Control data are from control animals on study [not baseline (pretreatment)] that have been sham dosed and experienced procedures as part of their designated study.

from the 28 selected studies were pooled, and mean, standard deviations and reference intervals for each outcome (biological) variable were calculated to allow characterisation of the effects of changes to housing and other predictor variables in this selected Mauritian population.

4.2.7a Reference intervals

Reference intervals are statistically constructed to include a range of probable values that lie between a pair of numbers within which 95% of animals are expected to lie and are therefore considered characteristic of a healthy or "normal"[6] population (Lumsden & Mullen 1978; Lumsden 2000).

4.2.7b Rank percentile method

The rank percentile method was used to determine reference intervals in accordance with the guidelines of the Expert Panel on Theory of Reference Values of the Internal Federation of Clinical Chemistry (see Sasse *et al* 2000). The rank percentile method is preferred as it does not rely on normality of data (Lumsden & Mullen 1978; Solberg 1983; 1993; Lumsden 2000; Harvey *et al* 2008). Values were ranked from smallest to largest and the 95% (0.025- 0.975), 90% (0.05-0.95), (median) 50% (0.5), (interquartile range) 25-75% (0.25-0.75) percentiles were calculated because they afford good characterisation of the data distributions (Harrison *et al* 1978). The reference limit is the value corresponding to the rank number.

4.2.7c Transformation of data to perform parametric statistics

The mean and standard deviation (Table 4.3.2) were calculated from transformed data (Section 4.2.6) by back transformation ($10^{\text{transformed mean}}$), therefore determining the true mean from the original data set. The standard deviation was calculated from the derived mean using the following equation:

$$\sigma = \sqrt{\frac{\sum(\chi - \mu)}{n-1}}$$ (Sum of squares divided by the number of observations -1)

[6] Note the term "normal" is inappropriate and may be misleading because it implies that all animals within the range are healthy or "normal" individuals, yet animals that lie outside of this range may be clinically normal. Also, severely sick animals might have blood analytes that lie within the historically constructed normal range (Hall 2007).

where σ is the standard deviation, χ is the value of the observation, μ is the population mean (from back transformed data or antilog), n-1 is the number of observations minus 1 (Fowler *et al* 1998, *p*36 - 37).

4.2.8 Effects of age and sex, and age*sex interactions

It is recommended that testing laboratories maintain historical data to characterise species, origin, sex and age-related variations (James 1993). Given that the CASE sponsor control data were not suitable for comparison, the effects of age, sex and age*sex interactions were determined using a two-way Analysis of Variance (ANOVA) on transformed data, for each biological (outcome) variable. A P value < 0.05 was considered significant.

4.2.9 Multiple linear regression

Multiple regression analysis was chosen because it allowed quantification of relationships between outcome variables; the selected core battery of biological variables of interest to toxicologist and welfare scientists alike (Chapters 1 & 3) and predictor variables, along with housing changes (Table 4.2.6) likely to affect macaque's biological data. It allowed prediction of effects of the dependent (outcome) variable from one or more independent (predictor) variables (Field 2005). The outcome variable (biological variables in the core battery) is predicted using the linear combination of predictors that correlate maximally. The relationship in its simplest terms can be described as:

$$Yi = (bo + bi\,Xi)$$

where *Yi* is the outcome variable*; Xi* is the predictor variable; *bi* is the gradient of the line fitted to the data*; bo* the intercept of the line*; bi* and *bo* are known as the regression coefficients and can be seen illustrated in Figure 4.2.1

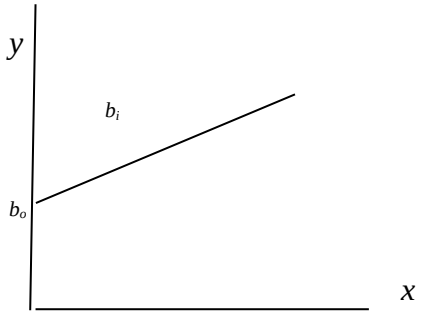


Figure 4.2.1 The relationship between outcome variable (*Yi*) and predictor (*Xi*) variable in a regression model. bo= Intercept of the line; b*i*= Gradient of the line fitted to the data.

A forward stepwise method was chosen over hierarchical and forced entry methods as it does not require a *priori* knowledge (e.g. prior work or background data from published literature) to select in order of importance which of the predictor variables would exert the greatest influence on the outcome (biological) variables (Field 2005). This PhD work is novel in its multivariate examination of the effects of housing on biological data from cynomolgus macaques. Instead, with the stepwise method, SPSS uses mathematical criteria to enter the predictor variables into the model in hierarchical order. The constant (bo) plus the predictor variable(s) that best predict the outcome (biological) variables are retained in the model.

Data were entered into SPSS as outlined in Table 4.2.6, with categorical data for housing (3 categories) being substituted for dummy data to allow SPSS to run analysis on only 2 categories at a time (model constraints) with the purpose-built gang-caging condition kept constant. Regression model, descriptive statistics, summary of model and model parameters (diagnostics) were selected and checked to ensure the model had conformed to selected model parameters and assumptions, allowing confident interpretation.

4.2.10 Analysis of Covariance (ANCOVA) with contrasts: Comparison between housing conditions

One or more continuous (covariates) that were found to significantly predict the outcome (biological) variable alongside housing condition were included in an analysis of Covariance (ANCOVA) with contrasts. A simple contrast with Sidak correction allowed for single and dog housing conditions to be compared to purpose-built housing (as with dummy variables entered into the regression model). A P value < 0.05 was considered significant.

4.3 Results

4.3.1 Data included for analysis

Macaques' biological data recorded as part of the core battery at baseline from twenty-eight studies, involving 840 animals, with equal numbers of males and females were reviewed (Table 4.2.2). Fourteen studies (N = 422 animals) were included from modified housing (A, Single: 4 studies; B, Dog: 10 studies) pre March 2003 and 14 (N = 418 animals) were from post March 2004 (C, purpose-built group housing). Control data from sham dosed animals (N = 164: N_A = 22; N_B = 54; Nc = 94) were included for analysis of organ: body weight ratios at end-of-life.

4.3.2 Animal and study demographics

Table 4.3.1 gives the demographic data for the studies that were included for analysis including arrivals, length of acclimatisation time before start of study and macaque age at start of study. These data are summarised by housing condition (Figures 4.3.1 &2).

Table 4.3.1 Animal and study demographics for 28 studies

Housing		Code	Same arrivals? Y/N	Age group	Age range (w)	Acclim range (w)
				All	**All**	**All**
Pre-2003 modified single & dog	A	1	Y	Juv: 1YA	60-136	4
	B	2	N; 2 arrivals 1w apart	Juv	82-132	20-21
	B	3	Y	Juv	70 - 120	11
	B	4	Y	Juv	70-108	10
	A	5	N; 2 arrivals 16w apart	Juv	60-144	16-36
	B	6	Y	Juv	74–95	6
	B	7	Y	Juv	86-102	12
	B	8	N; 3 arrivals 18w apart	Juv	74-118	18-36
	B	9	Y	Juv	74-108	17
	A	10	Y	Juv	63-95	9
	A	11	N; 4 arrivals 11w apart	Juv:5 YA	89-164	31-42
	B	12	N; 3 arrivals up to10w apart	Juv	73-93	9-17
	B	13	Y	Juv:2 YA	69-130	20
	B	14	N; 4 arrivals up to 18w apart	Juv	75 -126	16-34
Post-2004 purpose built gang	C	15	Y	YA	203-251	8
	C	16	N; 2 arrivals up to 44w apart	YA	169-229	11-44
	C	17	Y	YA	188-205	10
	C	18	N; 4 arrivals up to 25w apart	YA:8 Juv	78-190	4-29
	C	19	N; 2 arrivals up to 6w apart	YA	189-203	7-13
	C	20	N; 2 arrivals up to 2w apart	Juv	(60-115	11-13
	C	21	Y	Juv	55-108	18-26
	C	22	Y	YA	138-184	14
	C	23	N; 2 arrivals 6w apart	Juv	86-154	12-20
	C	24	N; 2 arrivals 1w apart	YA:3 Juv	117-180	11-12
	C	25	N; 4 arrivals up to 16w apart	YA:6 Juv	101-183	14-30
	C	26	Y	Juv	66-161	16
	C	27	Y	Juv	71-82	5
	C	28	N; 2 arrivals 3w apart	Juv	82-107	7-10
				Mean (w) (±SD) All	**113.9 (42.8)**	**15.1 (8.1)**
				Males	**113.0 (43.2)**	**15.3 (8.0)**
				Females	**114.8 (42.6)**	**15.0 (8.2)**

Housing condition: A Modified single; B Modified dog; C Purpose built gang; Code Assigned code for each study; All: All animals; Arrivals: Term used to describe the "batch" of animals arriving on a single shipment into the unit from Mauritius; Y: Yes; N: No; Age group: J Juvenile; YA Young Adult (Section 4.2.5i); The age of animals (weeks) at start of study (day 1 of dosing) as observed range(minimum/maximum); Acclim: Length of acclimatisaton time (weeks); time from arrival to day 1 of dose as observed range (minimum/maximum).

Figure 4.3.1 Mean age (in weeks; ±SD) of macaques at start of study for each housing condition

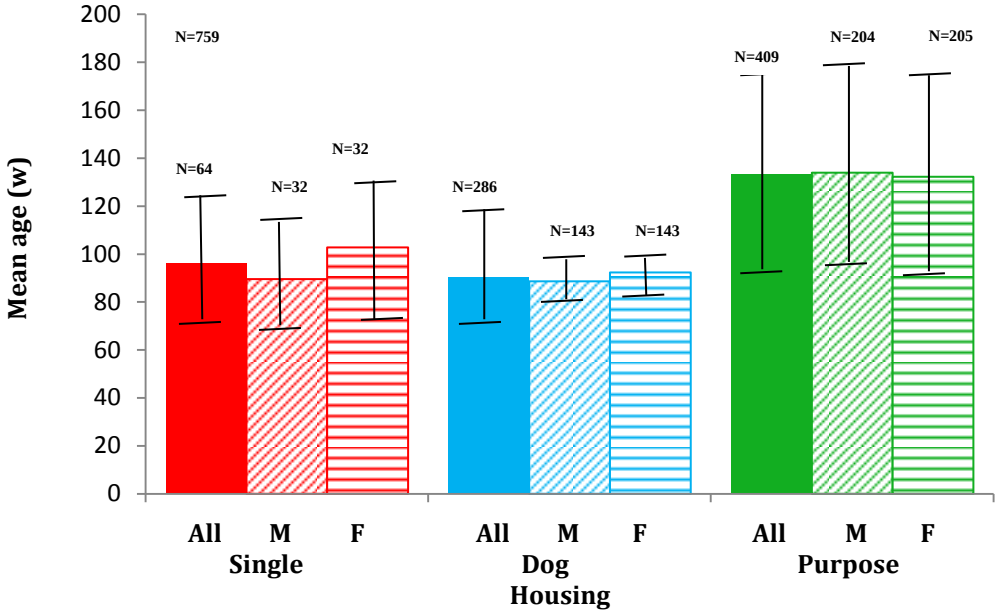
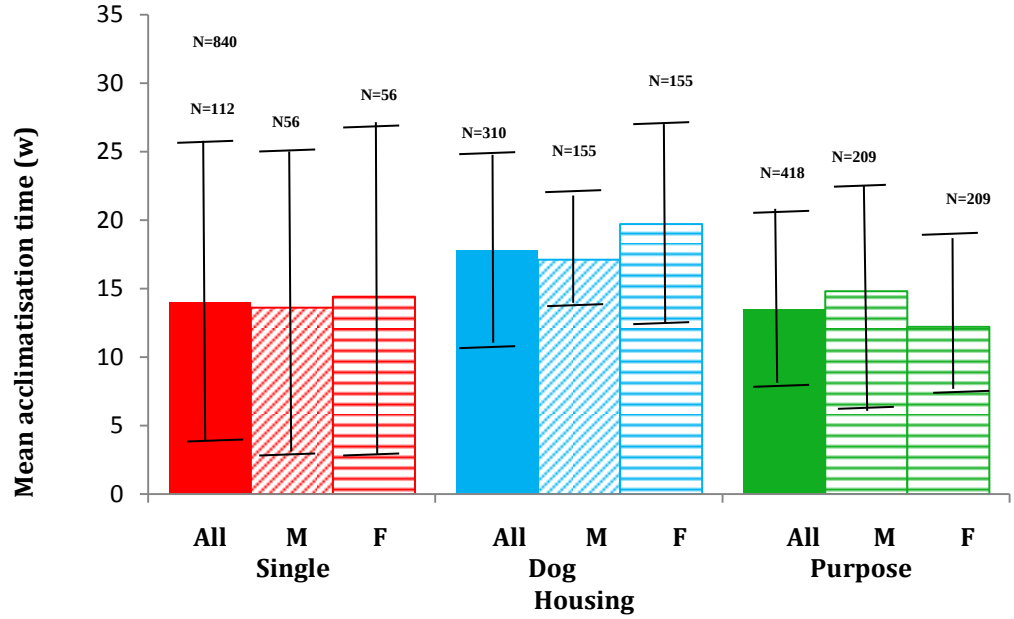


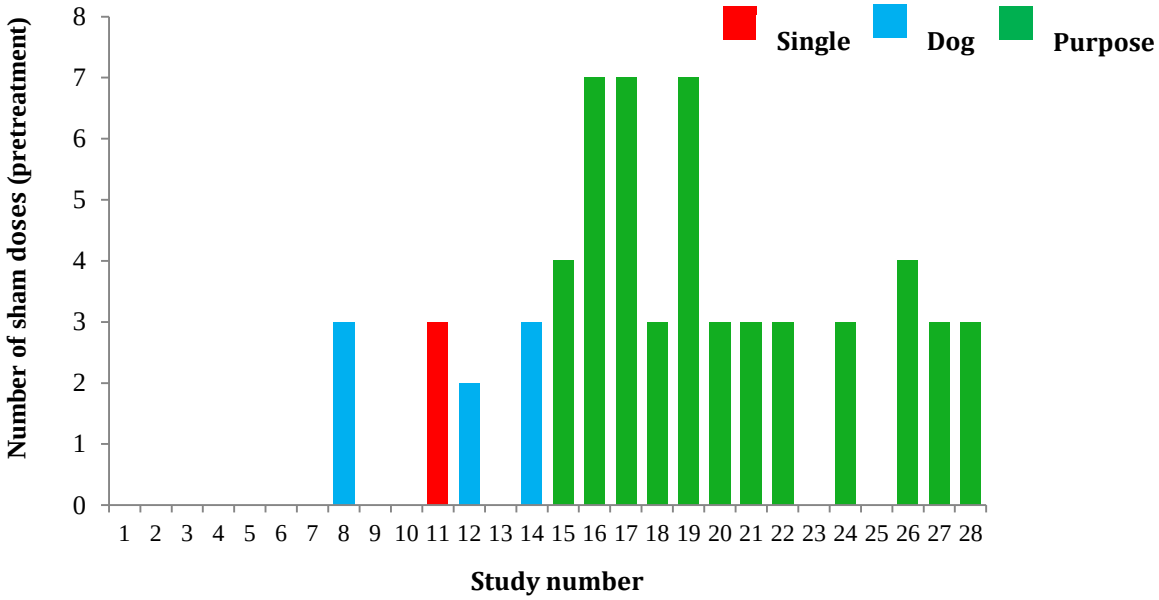
Figure 4.3.2 Mean acclimatisation time (in weeks; ±SD) of macaques at start of study for each housing condition



Macaques were older at the start of study in purpose-built gang-caging than in modified single or dog housing. The reasons for this are not clear; it was considered unlikely to be related to change in weaning policy at the breeders (personal communication CASE sponsor). The longer acclimatisation times seen in modified dog housing were due to disrupted transport arrangements during importation

of macaques from overseas breeding centres. As a consequence animals were brought into the CASE sponsor laboratory in large batches, ahead of known study requirements.

Figure 4.3.3 Number of sham doses[7] given to macaques during the pretreatment period before day 1 of dose in 28 studies



Sham dosing was not routinely undertaken in the modified single or dog housing conditions, but was performed regularly before onset of study in purpose-built gang-caging. The reasons for sham dosing cited by the CASE sponsor (personal communication) were firstly to ensure that individuals could be dosed by oral gavage without regurgitating some or all of the test dose (as it would be difficult to determine the quantity of dose an animal had received following regurgitation). Individuals that could not be successfully dosed were substituted prior to start of study. Further, as a secondary consideration, that macaques may habituate to handling, restraint and sensation of oral gavage with increasing experience before start of study (3 - 7 sham doses were performed).

[7] All animals are sham dosed once per day over consecutive days prior to day 1 of dosing. Sham dosing involves delivering a water bolus of known quantity directly into the stomach of macaques via a stomach tube, a dose route known as oral gavage. Animals are conscious and manually restrained in an upright position by a technician.

4.3.3 Characteristics of the overall population: Reference intervals

Reference intervals for biological data from male and female, juvenile and young adult (pooled) cynomolgus macaques of Mauritian origin mined from historic study data are displayed in Table 4.3.2. The standard deviation was used as a measure of variability of sample scores around the mean. Some biological variables in macaque baseline data showed greater variability than others (Table 4.3.2); haematological N, M, and AST, ALP clinical chemistry analytes, adrenal, pituitary and thyroid organ weights (% of body weight) have large standard deviations ≥50% relative to the mean. By contrast, Hb, RBC count, PCV, TPROT, GLOB and heart rate cluster more closely (≤10% relative to mean).

Table 4.3.2 Reference intervals of biological data for selected population of cynomolgus macaques of Mauritian origin

Biological (Outcome) variable	N	Mean (±SD)	Median (IQR)	O-R	90%	95%
Haematology						
Hb (g/dL)	834	13.5 (0.82)	13.5 (13.1-14.2)	11.3-16.3	13.5-15.0	12.0-15.3
RBC (mil/cmm)	834	6.88 (0.46)	6.88 (6.59-7.20)	5.28-8.52	6.16-7.69	6.03-7.86
PCV (%)	834	45.9 (3.22)	45.8 (43.7-48.1)	37.8-57.6	41.1-51.2	40.1-52.6
PLAT (1000/cmm)	835	385 (84.09)	386 (331-449)	197-203	272-542	248-574
WBC (10^9/L)	834	10.5 (4.04)	10.8 (8.4-12.4)	4.2-38.6	6.0-18.0	6.0-21.0
N (10^9/L)	834	5.4 (3.77)	5.2 (4.0-7.3)	1.0-35.7	2.3-12.3	1.9-15.1
L (10^9/L)	834	3.8 (1.67)	4.0 (3.5-5.0)	1.1-9.6	2.0-7.2	0.5-7.9
M (10^9/L)	834	0.3 (0.15)	0.3 (0.3-0.4)	0.1-1.2	0.1-0.6	0.1-0.7
Clinical chemistry						
AST (IU/L)	824	36 (38.21)	35 (31-43)	16-100	23-61	22-68
ALT (IU/L)	824	42 (18.02)	42 (34-54)	12-211	23-73	21-83
GGT (IU/L)	750	122 (41.84)	128 (101-153)	42-316	65-204	59-224
ALP (IU/L)	824	1572 (794.10)	1682 (118-2216)	430-5990	647- 3129	572-3555
Urea (mmol/L)	823	6.5 (1.67)	6.8 (5.7-8.0)	3.1-12.5	4.2-9.7	3.9-10.5
TBILI (mmol/L)	781	4.5 (2.19)	4.5 (3.5-5.9)	1.4-16.4	2.3-9.4	2.1-10.2
CREAT (mmol/L)	824	81 (19.37)	84 (71-97)	28-137	51-115	46-121
TPROT (g/L)	824	85 (4.66)	85 (83-89)	69-104	78-93	77-95
GLOB (g/L)	637	38 (3.93)	39 (36-42)	28-51	32-45	31-47
Heart rate						
HR (bpm)	824	241 (24.20)	244 (229-259)	129-311	197-275	188-280
Organ:body weight ratios						
Adrenals (%)	164	0.023 (0.028)	0.022 (0.026-0.990)	0.013-0.327	0.014-0.038	0.035-0.983
Kidneys (%)	170	0.433 (0.062)	0.441 (0.010-0.475)	0.044-0.574	0.005-0.537	0.559-0.349
Spleen (%)	104	0.224 (0.059)	0.225 (0.253-0.752)	0.127-0.425	0.131-0.365	0.145-0.410
Liver (%)	170	2.114 (0.253)	2.118 (1.947-2.282)	1.243-3.078	1.563-2.559	1.682-2.721
Pituitary (%)	103	0.002 (0.001)	0.002 (0.002-0.002)	0.001-0.009	0.001-0.003	0.001-0.006
Thyroid (%)	98	0.010 (0.008)	0.010 (0.008-0.020)	0.003-0.084	0.005-0.016	0.006-0.018

N: Number of animals included for analysis; Median: 50% (0.5); IQR: Interquartile range 25-75% (0.25-0.75); 90% (0.05-0.95); 95% (0.025-0.975) percentiles; O-R: Observed range (min-max).

4.3.4 Effects of age and sex on cynomolgus macaques' biological data

Table 4.3.3 gives age and sex differences in haematology and clinical chemistry analytes, heart rate, and organ:body weight ratio reference intervals derived from historic baseline data of cynomolgus macaques.

Only three biological variables; WBC count, pituitary and thyroid organs did not show significant variations on the basis of either gender or age group. However, with the inclusion of predictor variables in a multiple regression model age was found to have a small, but significant effect (0.7%) on WBC counts. Neutrophil counts follow a similar pattern, age accounted for 0.7% of the variation, but were not significant when age, sex, and age*sex interactions were examined without controlling for other predictor variables. Sex was excluded from PCV and liver:body weight ratios and age for PLAT counts and heart rate by the regression model, as they were not found to have a significant effect on variation

Table 4.3.3 Age and sex related variation in historic baseline biological data of cynomolgus macaques of Mauritian origin

Analyte	Pairwise comparisons							
	Juv	YA	M	F	MJuv	MYA	FJuv	FYA
Haematology								
Hb (g/dL)	13.5^{ns} 0.8	13.5 0.9	**13.7***** 0.8	13.4 0.8	13.7 0.8	**13.9***** 0.8	**13.5***** 0.8	13.1 0.8
RBC (mil/cmm)	**6.92***** 0.45	6.75 0.28	**6.92***** 0.46	6.84 0.46	**6.93***** 0.46	6.89 0.44	**6.92***** 0.43	6.62 0.48
PCV (%)	30.0 3.1	**45.6***** 3.5	**46.1***** 3.2	45.7 3.2	**45.8***** 3.2	46.8 3.4	**46.1***** 3.1	44.6 3.3
PLAT (1000/cmm)	**389*** 85.1	372 79.8	385^{ns} 85.5	385 82.3	**390*** 87.2	370 78.9	388^{ns} 83.2	375 80.8
WBC (10^9/L)	10.6^{ns} 4.3	10.3 3.3	10.5^{ns} 4.2	10.5 3.9	10.6^{ns} 4.4	10.4 3.4	10.6^{ns} 4.1	10.2 3.2
N (10^9/L)	5.4^{ns} 4.0	5.5 3.0	5.2 3.9	**5.6*** 3.7	5.2^{ns} 4.1	5.1 3.1	5.5^{ns} 3.9	5.9 3.0
L (10^9/L)	3.8^{ns} 1.7	3.8 1.4	**4.0***** 1.7	3.6 1.7	4.0^{ns} 1.8	4.2 1.4	3.7^{ns} 1.7	3.4 1.2
Clinical chemistry								
AST (IU/L)	**38***** 10.8	32 10.1	36^{ns} 37.5	37 13.0	37^{ns} 10.8	32 33.1	39^{ns} 13.3	31 9.8
ALT (IU/L)	**44***** 15.6	37 23.6	42^{ns} 16.2	42 19.6	**44***** 16.2	35 14.3	**43***** 15.0	38 30.0
Gamma GT (IU/L)	**137***** 39.7	90 34.2	**135***** 41.6	110 38.4	**145***** 40.3	112 33.8	**130***** 30.1	73 18.8
ALP (IU/L)	**1752***** 789.2	1118 516.2	1676 754.5	1474*** 825.9	**1746***** 811.9	1462 404.2	**1758***** 766.6	871 440.4
UREA (mmol/L)	6.5 ^{ns} 1.7	6.5 1.5	**6.7**** 1.6	6.4 1.7	6.6^{ns} 1.8	6.7 1.3	6.4^{ns} 1.7	6.3 1.7
CREAT (mmol/L)	74 15.5	**104***** 13.6	81^{ns} 19.7	81 19.0	74 15.0	**106*** 13.3	74 16.1	**102*** 12.1
T PROT (g/L)	84 4.1	**89***** 4.6	85^{ns} 4.8	85 4.5	84 4.0	**88***** 4.6	84 4.3	**88***** 4.4
GLOB (g/L)	**38***** 3.9	40 3.7	38^{ns} 4.0	38 3.9	38^{ns} 3.9	40 3.8	40^{ns} 3.8	38 3.8
Heart rate								
HR (bpm)	**243*** 23.0	237 25.3	240^{ns} 25.0	240 21.4	**242*** 23.7	237 20.2	**242*** 26.2	238 22.5
Organ:body weight ratios								
Adrenals (%)	**0.030***** 0.003	0.020 0.003	0.022 0.003	**0.025*** 0.003	0.024^{ns} 0.003	0.020 0.003	0.026^{ns} 0.003	0.022 0.003
Kidneys (%)	**0.445**** 0.001	0.404 0.041	0.438^{ns} 0.051	0.426 0.069	**0.458**** 0.047	0.399 0.035	**0.454**** 0.075	0.409 0.047
Spleen (%)	**0.237**** 0.058	0.173 0.031	0.229 0.058	0.220 0.063	**0.246**** 0.054	0.170 0.025	**0.230**** 0.063	0.177 0.037
Liver (%)	**2.228**** 0.223	1.933 0.163	**2.113**** 0.275	2.145 0.219	**2.239**** 0.235	1.869 0.153	**2.217**** 0.211	1.999 0.150
Pituitary (%)	0.002^{ns} 0.001	0.002 0.001	0.002^{ns} 0.001	0.002 0.001	0.002^{ns} 0.001	0.002 0.001	0.002^{ns} 0.001	0.002 0.001
Thyroid (%)	1.029^{ns} 1.020	1.029 1.020	1.029^{ns} 1.020	1.029 1.020	1.029^{ns} 1.020	1.029 1.020	1.029^{ns} 1.020	1.029 1.020

Quoted: Mean±SD; Pairwise comparison of means: $p<0.05$ was considered significant; * p <0.05 (0.01-0.05); ** p < 0.01; *** $p<0.005$; ns: not significant. Juv: Juvenile (aged 55–129w); YA: Young adult (aged 130-251w); M: Males; F: Females.

Males (M) significantly > Females (F); F significantly > M; Juveniles (Juv) significantly >young adults (YA); YA significantly > Juv; Not significant.

4.3.5 The effects of multiple 'predictors' on variation in baseline biological data of cynomolgus macaques

Housing was found to have a significant effect on the variability of macaque baseline biological data in all but two (monocytes and total bilirubin) clinical pathology parameters. Body weight change recorded over the two week pretreatment period prior to day 1 of dose, and heart rates, and liver:body weight ratios were also significantly affected by housing condition (Table 4.3.4; Figure 4.3.4). Thyroid and pituitary organs did not show variation with respect to any of the 'predictor' variables examined and those data are not reported.

The percentage variation (Table 4.3.4) in macaque baseline data accounted for by housing in combination with other 'predictor' variables was different for each biological variable. The total variation that could be accounted for by 'predictor' variables are quoted in the overall model (Table 4.3.4) and for housing only in Figure 4.3.4. Housing condition was the only 'predictor' variable found to have a significant effect on macaque PLAT counts (4.0%) in the regression model. Whilst it accounted for the largest variations out of all predictors for WBC (3.0%) and N (5.3%) counts, ALT (4.6%) and urea (2.9%) concentrations, heart rate (9.7%), and body weight change (6.5%). In comparison to other predictor variables entered into the multiple regression model, housing, whilst having a significant effect, accounted for the smallest proportion of variation in macaque L count (0.5%), GGT (1.2%), ALP (0.5%), TPROT (1.0%), and GLOB (2.1%) concentrations.

Table 4.3.4 Regression model output: Quantifying variability in the baseline biological (outcome) data from cynomolgus macaques

Biological (outcome variable)	Predictors	Model summary		Model parameters			Significance of model (ANOVA)	
		R^2	%	B	SE	β	df	F
Haematology								
Hb	Constant			1.15	0.00	-	3	18.36***
(g/dL)	Sex	0.04***	4.2	-0.01	0.00	0.20***		
	Housing:Purpose-dog	0.06**	1.4	-0.01	0.00	-0.17***		
	Age	0.08**	1.6	-0.00	0.00	-0.14***		
Overall model *$R^2(3)=0.08$;8%***								
RBC	Constant			0.87	0.0	-	3	24.56***
(mil/cmm)	Age	0.06***	5.9	0.00	0.00	-0.31***		
	Housing:Single-dog	0.08***	2.4	-0.01	0.00	-0.17***		
	Sex	0.09**	1.1	0.01	0.00	-0.11**		
Overall model *$R^2(3)=0.09$;9%***								
PCV	Constant			1.65	0.01	-	3	15.22***
(%)	Sham dosing	0.03***	3.1	-0.00	0.00	-0.23***		
	Housing:Purpose-single	0.05**	1.4	0.14	0.00	0.13***		
	Age	0.05**	0.9	0.01	0.00	0.12*		
Overall model *$R^2(3)=0.05$;5%***								
PLAT	Constant			2.57	0.01	-	2	14.86***
(1000/cmm)	*Housing*:							
	Purpose-dog	0.03***	3.0	0.04	0.00	0.20***		
	Purpose-single	0.04***	1.0	0.03	0.01	0.08*		
Overall model *$R^2(2)=0.04$*;4%***								
WBC (10^9/L)	Constant			1.07	0.02	-	3	9.26***
	Housing:							
	Purpose-dog	0.02***	2.4	-0.06	0.01	-0.22***		
	Purpose-single	0.03*	0.6	-0.05	0.02	-0.11**		
	Age	0.04*	0.7	-0.00	0.00	-0.09*		
Overall model *$R^2(3)=0.04$*;4%*								
N	Constant			0.83	0.03		3	14.95***
(10^9/L)	*Housing*:							
	Purpose-dog	0.03***	3.3	-0.12	0.02	-0.26***		
	Purpose-single	0.05***	2.0	-0.13	0.03	-0.167***		
	Age	0.06*	0.7	0.00	0.00	-0.09*		
Overall model *$R^2(3)=0.06$*;6%*								
L	Constant			4.18	0.18	-	4	9.98***
(10^9/L)	Sham dosing	0.03***	3.0	-0.16	0.02	0.23***		
	Sex	0.04**	1.1	-0.34	0.12	-0.11**		
	Age	0.05*	0.7	0.00	0.00	0.10*		
	Housing:Purpose-single	0.05*	0.5	0.41	0.21	0.07*		
Overall model *$R^2(4)=0.05$*;5%*								
Clinical chemistry								
AST	Constant			1.62	0.02	-	5	23.84***
(IU/L)	Age	0.07***	7.2	-0.00***	0.00	-0.22		
	Housing:Purpose-single	0.11***	3.4	0.04**	0.02	0.10		
	Acclim	0.13***	2.1	0.00**	0.00	0.18		
	Sham dosing	0.13*	0.7	-0.01**	0.00	-0.16		
	Housing:							
	Purpose-dog	0.15**	1.2	-0.04**	0.01	0.14		
Overall model *$R^2(5)=0.15$;15%***								
ALT	Constant			1.66	0.02		3	20.01***
(IU/L)	*Housing*:Purpose-single	0.04***	4.3	0.09	0.02	0.16***		
	Age	0.07***	2.8	-0.01	0.00	-0.17***		
	Acclim	0.08*	0.9	0.02	0.00	0.10*		
Overall model *$R^2(3)=0.08$*;8%*								
GgT	Constant			2.37	0.02	-	5	137.10**
(IU/L)	Age	0.36***	36.1	-0.00	0.00	-0.63***		*
	Sex	0.45***	8.8	-0.08	0.01	-0.29***		
	Acclim	0.51***	6.2	0.01	0.00	0.29***		
	Housing:							
	Purpose-dog	0.52***	0.9	-0.03	0.01	-0.09**		
	Purpose-single	0.52*	0.3	0.04	0.02	-0.06*		
Overall model *$R^2(5)=0.52$*;52%*								
ALP	Constant			3.23	0.03		5	96.22***

(IU/L)	Acclim	0.20***	19.8	0.01	0.00	0.42***		
	Age	0.34***	15.8	-0.01	0.00	-0.26***		
	Sham dosing	0.38***	2.4	-0.02	0.00	-0.17***		
	Sex	0.40***	2.2	-0.07	0.01	-0.15***		
	Housing:Purpose-dog	0.41*	0.5	0.04	0.02	0.08*		
Overall model *R^2(5)=0.41*;41%*								
UREA	Constant			0.83	0.11		2	12.69***
(mmol/L)	*Housing*:Purpose-single	0.03***	2.9	0.06	0.01	0.17***		
	Sex	0.04*	0.6	-0.02	0.01	-0.08*		
Overall model *R^2(2)=0.04*;4%*								
TBILI	Constant			0.64	0.01	-	1	9.38**
(mmol/L)	Age	0.01*	1.3	0.04	0.01	0.12**		
Overall model *R^2(1)=0.01*;1%*								
CREAT	Constant			29.29	18.90	-	4	270.19**
(mmol/L)	Age	0.49***	49.4	0.43	0.01	0.88***		*
	Housing:							
	Purpose-dog	0.56***	6.1	15.50	1.21	0.36***		
	Purpose-single	0.61***	5.0	18.13	1.88	0.26***		
	Acclim	0.61**	0.2	-0.13	0.07	-0.05**		
Overall model *R^2(4)=0.61;61%***								
TPROT	Constant			1.91	0.00	-	3	78.90***
(g/L)	Age	0.20***	20.0	0.00	0.00	0.47***		
	Acclim	0.24***	4.3	-0.00	0.00	-0.23***		
	Housing:Purpose-single	0.25*	1.0	0.01	0.00	0.10**		
Overall model *R^2(3)=0.25*;25%*								
GLOB	Constant			33.70	0.85	-	4	31.84***
(g/L)	Sham dosing	0.07***	7.4	-0.68	0.15	-0.25***		
	Age	0.18***	10.2	0.05	0.01	0.37***		
	Housing:							
	Purpose-single	0.18*	0.8	2.00	0.58	0.16**		
	Purpose-dog	0.20**	1.3	1.34	0.47	0.17**		
Overall model *R^2(4)=0.20*;20%***								
Heart rate								
HR	Constant			2.36	0.00	-	3	29.11***
(bpm)	*Housing*:							
	Purpose–dog	0.04***	4.4	0.02	0.04	0.23***		
	Purpose–single	0.10***	5.3	0.03	0.01	0.20***		
	Acclim	0.11*	1.2	0.00	0.00	0.12*		
Overall model *R^2(3)=0.11*;11%*								
Body weight change								
BWC	Constant			3.50	0.55		4	20.77***
(%)	*Housing:*Purpose-single	0.03***	3.3	1.74	0.56	0.13		
	Acclim	0.06***	2.3	-0.05	0.02	-0.10		
	Age	0.07***	1.6	-0.02	0.00	-0.22		
	*Housing:*Purpose-dog	0.10***	3.2	-1.78	0.35	-0.21		
Overall model *R^2(4)=0.10*;10%***								
Organ:Body weight ratio								
Adrenals (%)	Constant			-1.53	0.04	-	2	16.28***
	Age	0.12***	11.5	-0.00	0.00	-0.34		
	Sex	0.19***	7.4	0.09	0.03	0.27		
Overall model *R^2(2)=0.19*;19%***								
Kidneys	Constant			-0.30	0.02		1	13.55***
(%)	Age	0.09***	9.0	-0.00	0.00	-0.3**		
Overall model *R^2(1)=0.09*;9%***								
Spleen	Constant			-0.51	0.04	-	2	14.06***
(%)	Age	0.22***	21.9	-0.00	0.00	-0.48***		
	Sham dosing	0.27**	4.9	0.01	0.01	0.22*		
Overall model *R^2(2)=0.27*;27%***								
Liver	Constant			0.38	0.01		3	44.55***
(%)	Age	0.39***	39.2	-0.00	0.00	-0.62		
	Housing:Purpose-dog	0.45***	6.0	-0.39	0.01	0.34		
	Sham dosing	0.48**	0.3	0.01	0.00	0.24		
Overall model *R^2(3)=0.48; 48%***								

Data given to two decimal places. R^2 (*df*) is a measure of how much variability in the biological (outcome) variable is accounted for by the predictors selected by SPSS to be included in the model. The significance of the model at predicting the biological (outcome) variable is determined in the model using ANOVA. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

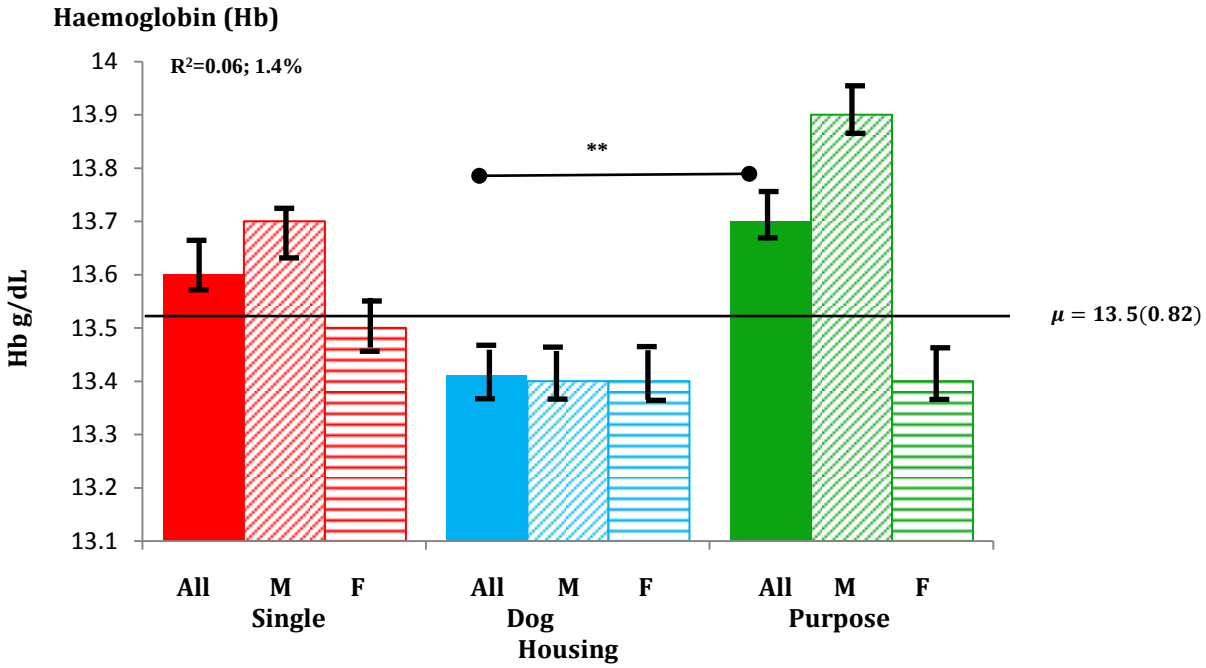
The variance explained by the overall multiple regression model (R^2;%) was wide-ranging; 1% (TBILI)-61% (CREAT) of the variance in macaques' biological data was explained by combination of predictor variables. Conversely, between 49%-99% of variation was not explained by the model, a sizeable proportion, not identified in historic baseline data of macaques.

4.3.6 The effect of housing on baseline biological data of cynomolgus macaques

The effect of housing on baseline biological data is shown in Figures 4.3.4 a - e

Figure 4.3.4 The effect of housing on baseline biological data of cynomolgus macaques: Haematology, clinical chemistry analytes, heart rate, body weight change and organ:body weight ratio.

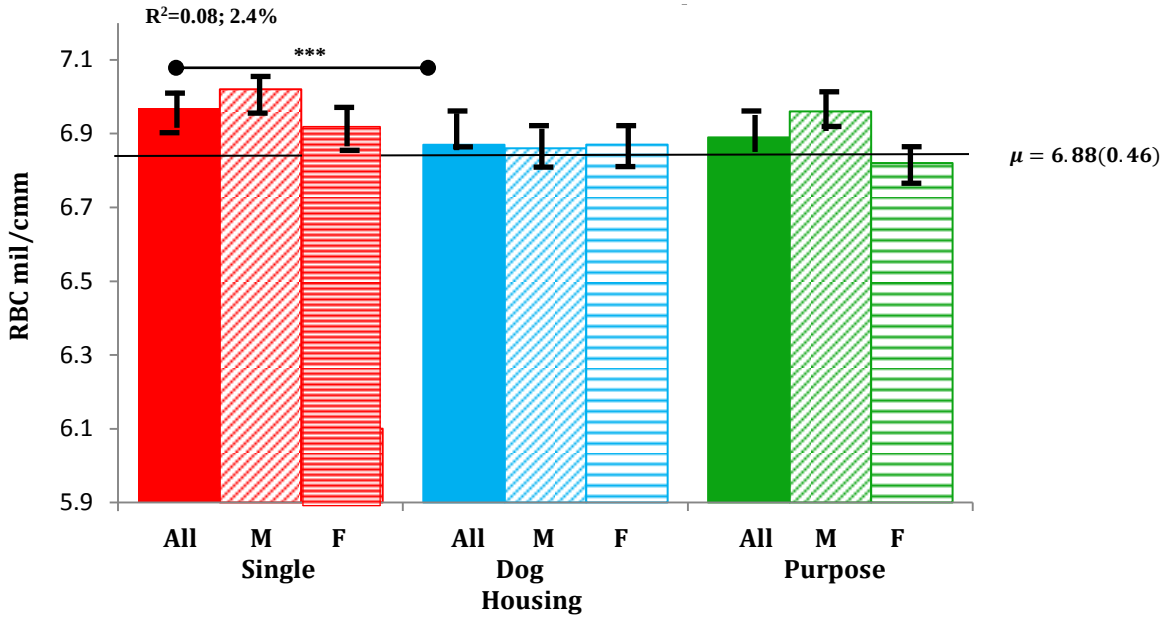
(a) Haematology



Housing had a significant effect on variation in haemoglobin levels (R^2=0.06) after controlling for sex, $F(2,829)=5.65$, $p<0.01$. Macaques housed in modified dog housing had significantly lower Hb levels compared to those in purpose-built gang-caging, $t(829)=-3.36$, $p<0.01$, $r=0.03$.

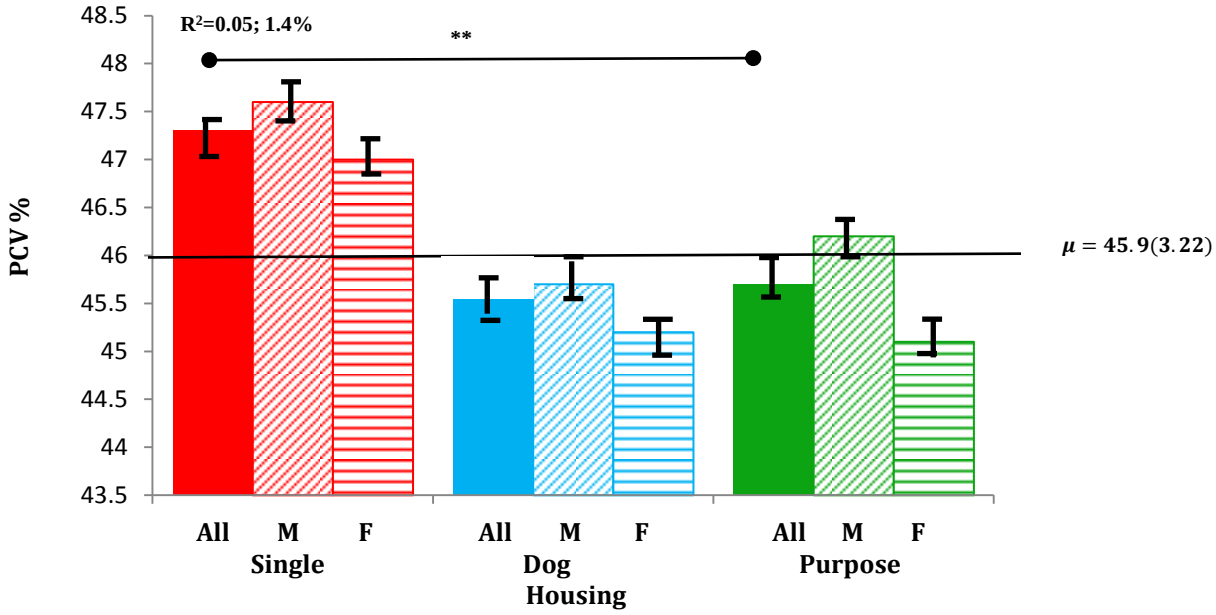
R^2 is a measure of how much variability in macaque biological (outcome) data are accounted for by housing condition; % variation in the biological (outcome) data are accounted for by housing condition; μ is the population mean (±SD) calculated as a reference interval from the entire population (Table 4.3.2); ANCOVA with simple contrasts between housing condition indicate the significant difference between housing conditions; single and dog are compared to purpose-built; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Red blood cells (RBC)



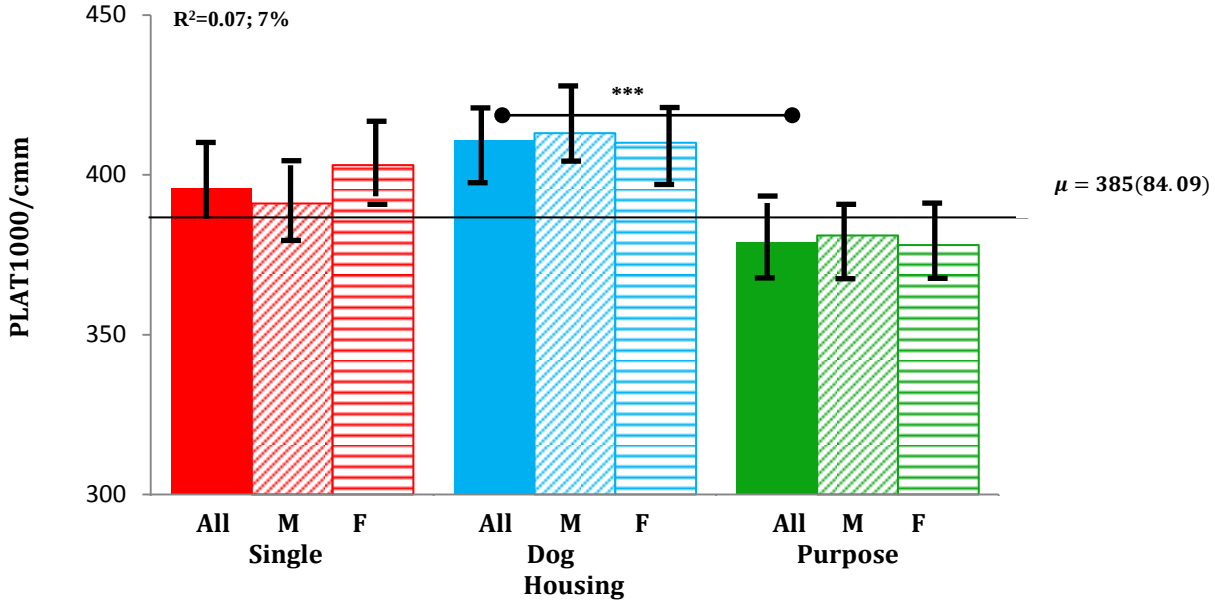
After controlling for sex and age, housing condition was found to have a significant effect on RBC count, $F(2,708)=9.45$, $p<0.001$. Red blood cell counts were lower in macaques housed in modified dog pens compared to single cages, $t(708)=-4.281$, $p<0.001$, $r=0.09$.

Packed cell volume (PCV)



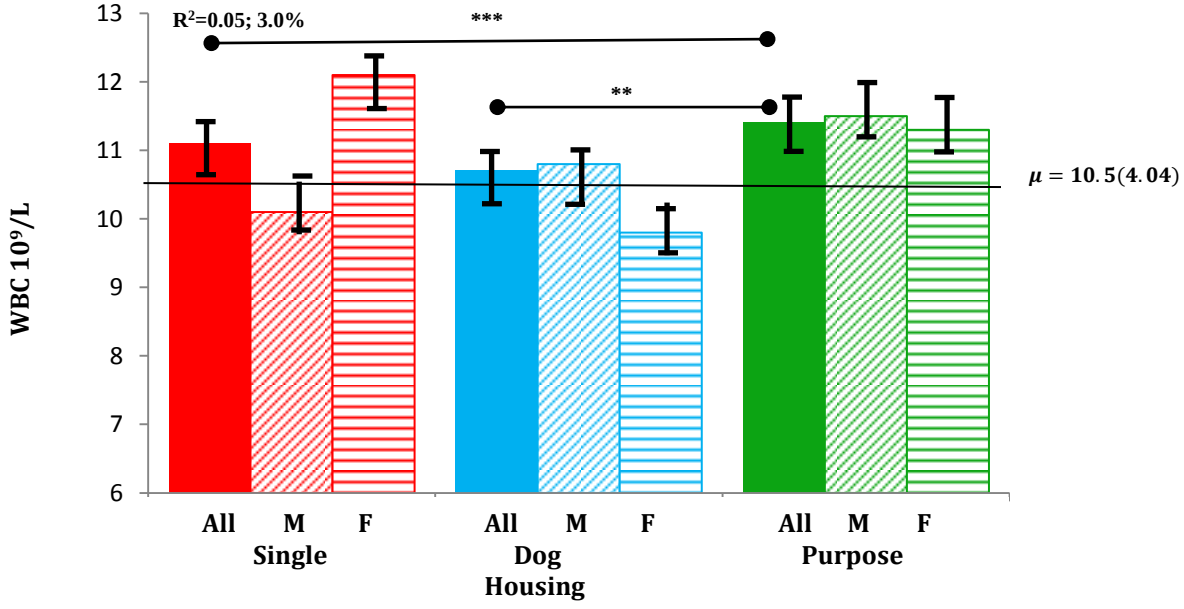
Housing significantly affected the variation in macaques' PCV after controlling for age and number of sham doses, $F(2,708)=6.57$, $p<0.001$. Macaques housed in modified single cages had significantly higher PCV than those in purpose built gang-caging, $t(708)=2.48$, $p0.05$, $r=0.05$.

Platelets (PLAT)



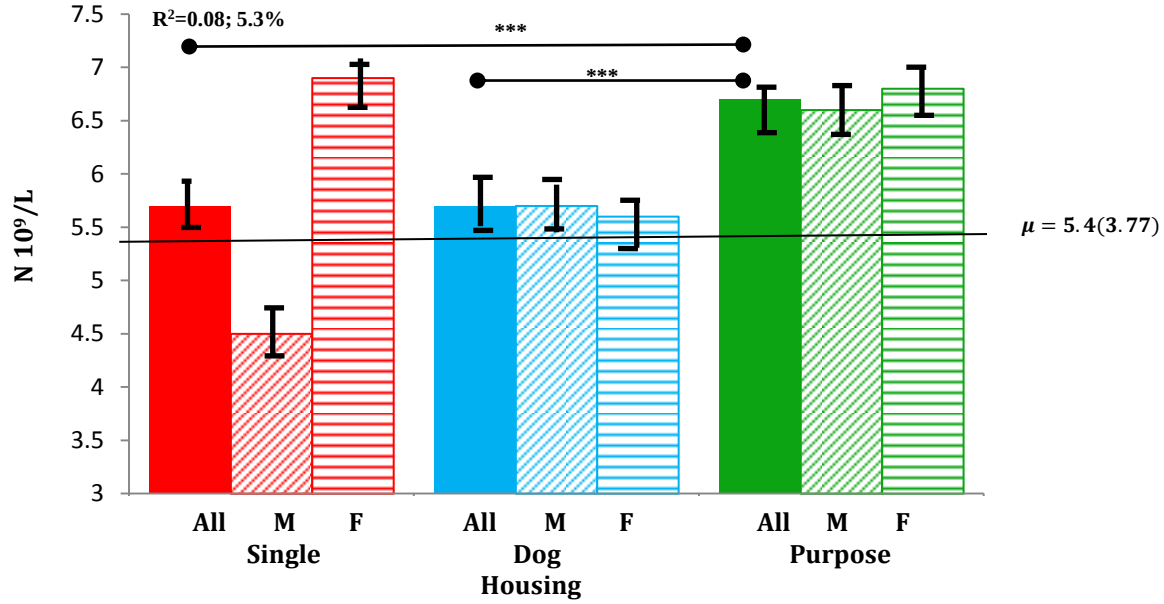
Platelet levels were significantly affected by housing condition, $F(2,828) = 14.69$, $p<0.001$. Macaques housed in purpose-built accommodation had significantly lower PLAT counts than those housed in modified dog pens, $t(828)=5.40$, $p<0.001$, $r=0.03$.

White blood cells (WBC)



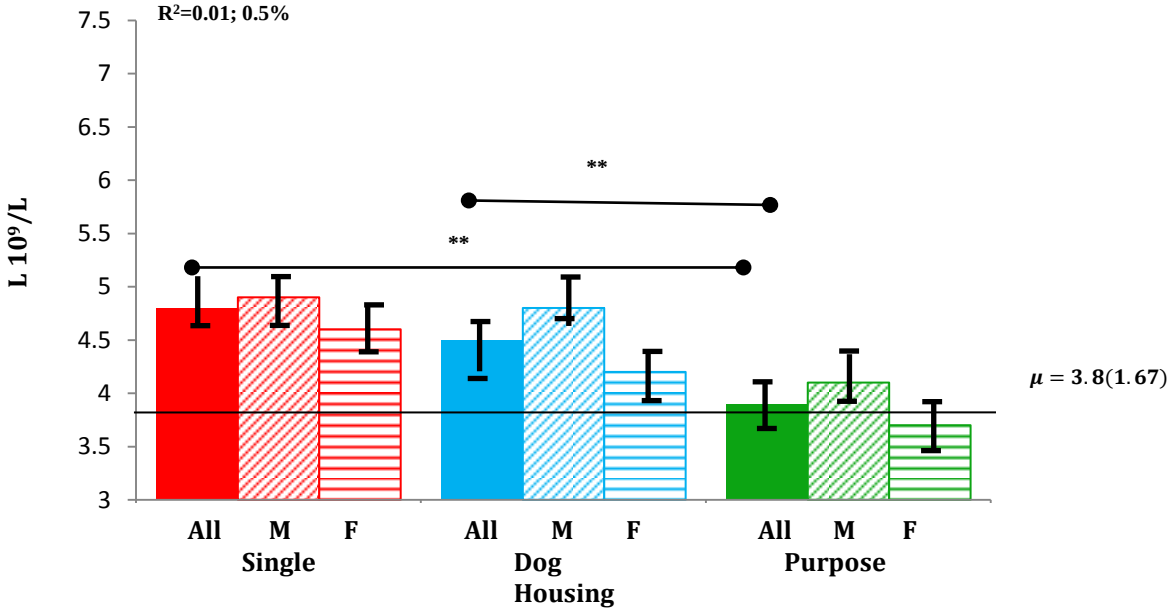
After controlling for age, housing was found to have a significant effect on WBC counts, $F(2,709)=13.88$, $p<0.001$. White blood cell counts were significantly higher in macaques housed in purpose built gang-caging compared to modified single, $t(709)=-5.14$, $p<0.001$, $r=0.34$, and dog pens, $t(709)=-2.67$, $p<0.01$.

Neutrophils (N)



Neutrophil counts were found to be significantly affected by housing condition after controlling for age, $F(2,708)=22.03$, $p<0.001$. Both the modified single, $t(708)=-4.31$, $p<0.001$ and dog, $t(709)=-6.13$, $p<0.01$, $r=0.06$ housing conditions gave rise to significantly lower N counts compared to macaques housed in purpose-built gang accommodation.

Lymphocytes (L)

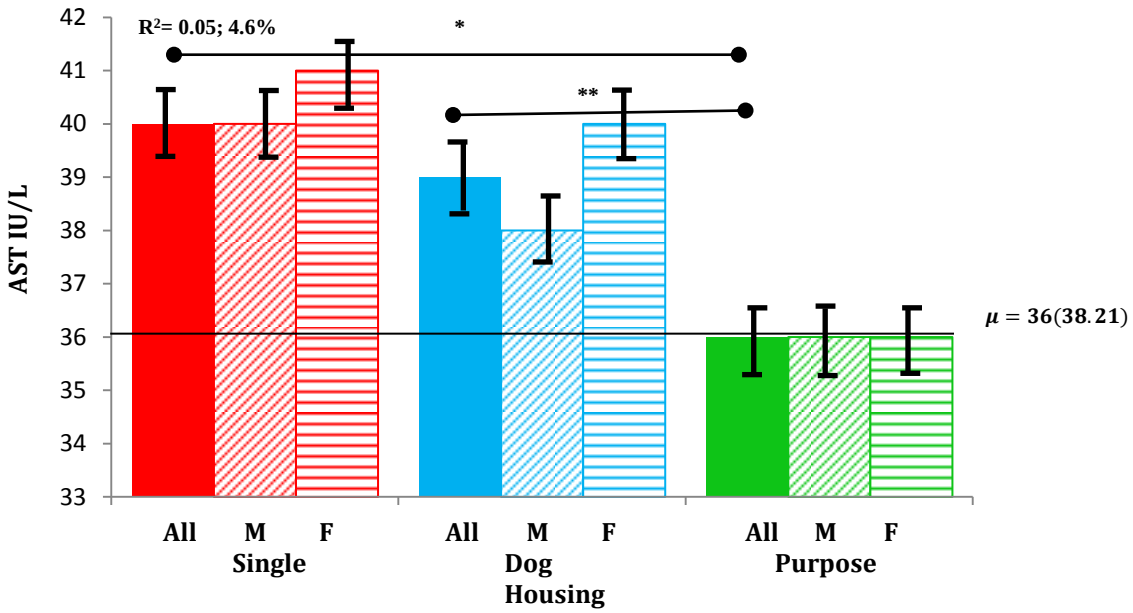


When controlled for sex, age and number of sham doses, housing was found to have an effect on lymphocyte counts, $F(2,706)=7.02$,p,0.00. Both modified single and dog housing were associated with

higher lymphocyte counts than macaques housed in purpose-built gang; single, $t(706)=3.15$, $p<0.01$; dog, $t(706)=3.02$, $p<0.01$, $r=0.07$.

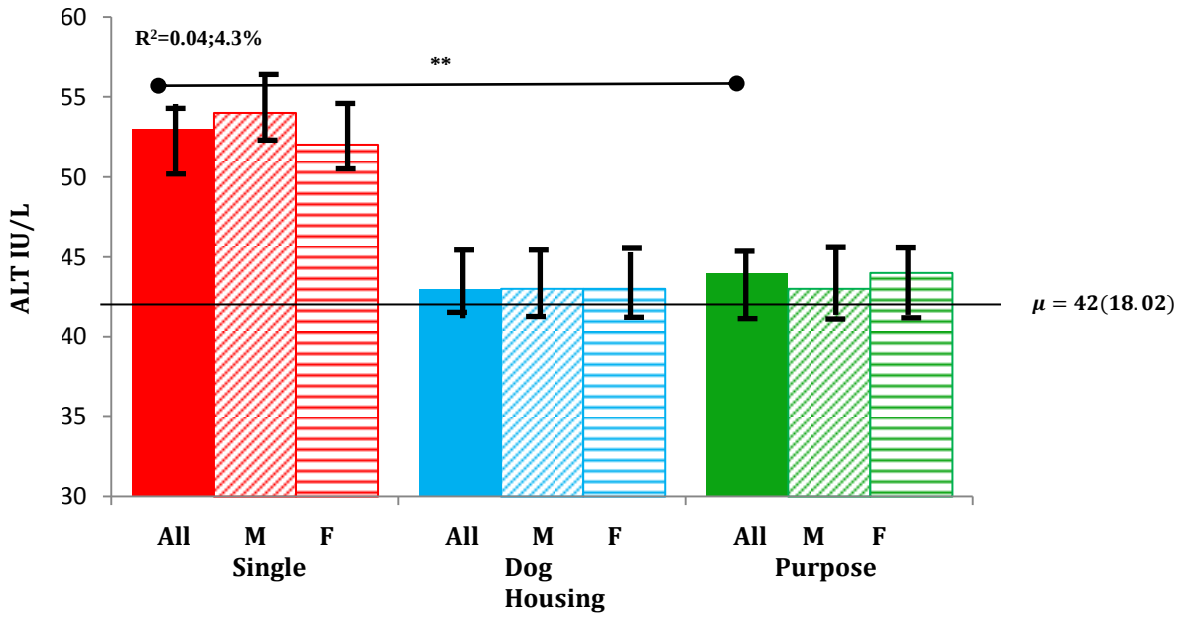
(b) Clinical chemistry

Aspartate aminotransferase (AST)

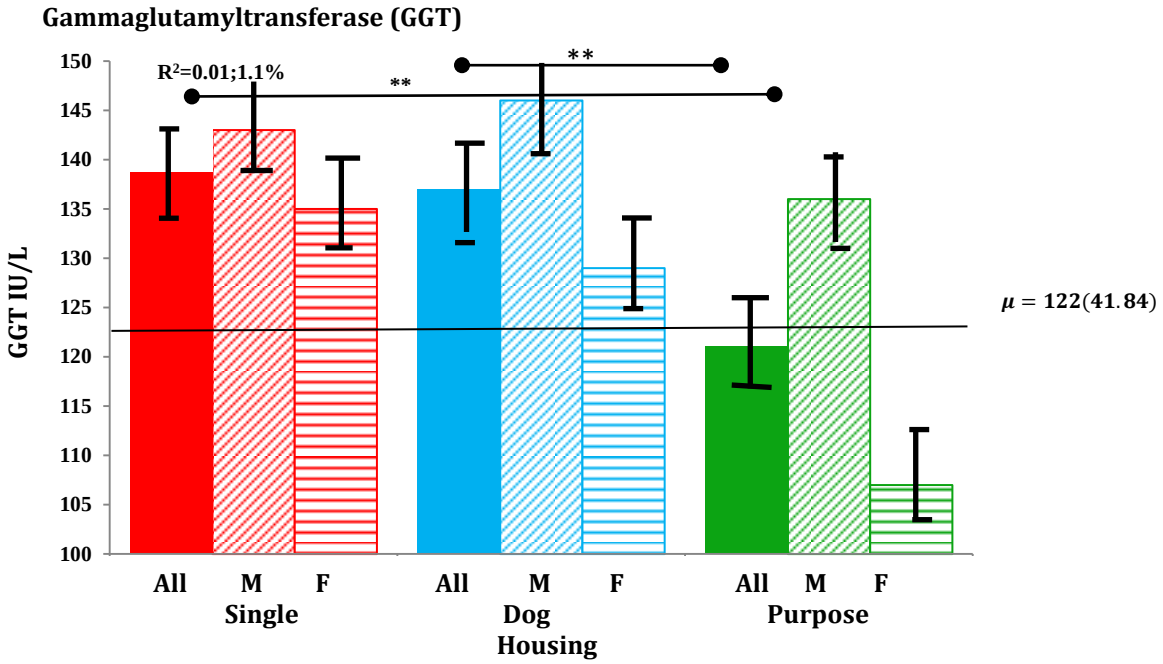


Housing was found to have a significant effect on AST levels, $F(2,697)=13.04$, $p<0.001$ when controlled for age, acclimatisation time and number of sham doses. Aspartate aminotransferase levels were elevated in macaques housed in modified single and dog housing conditions compared to those in purpose-built gang; single, $t(697)=2.42$, $p<0.05$ and dog, $t(697)=3.09$, $p<0.01$, $r=0.14$.

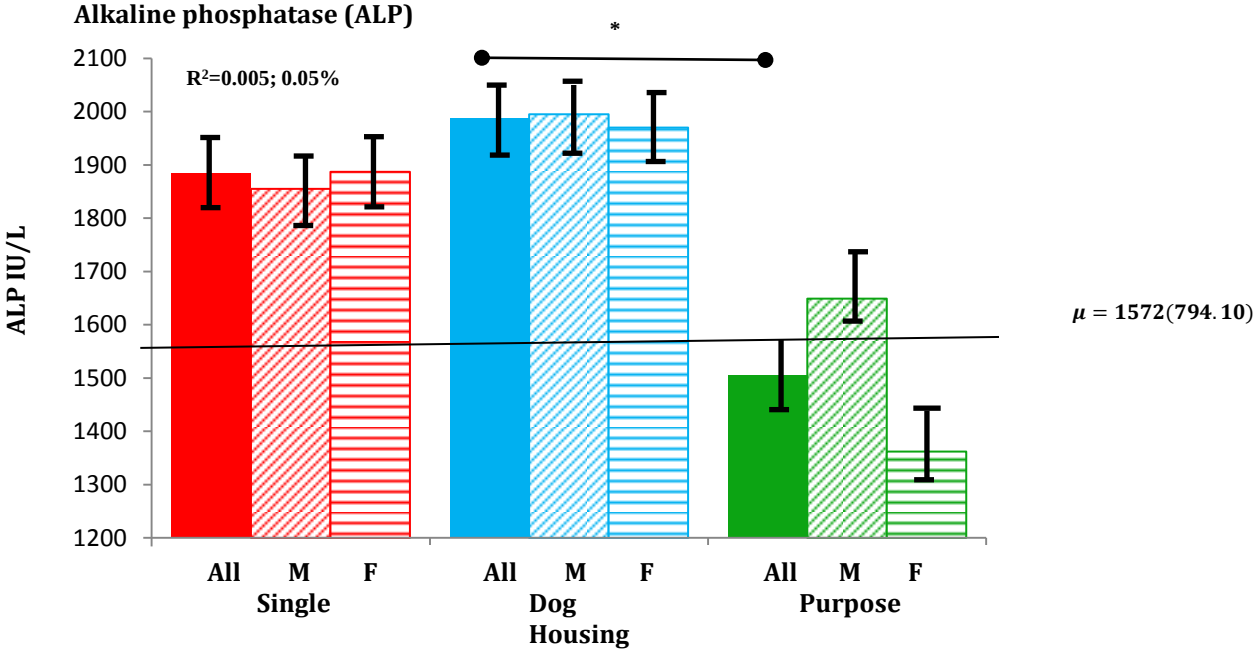
Alanine aminotransferase (ALT)



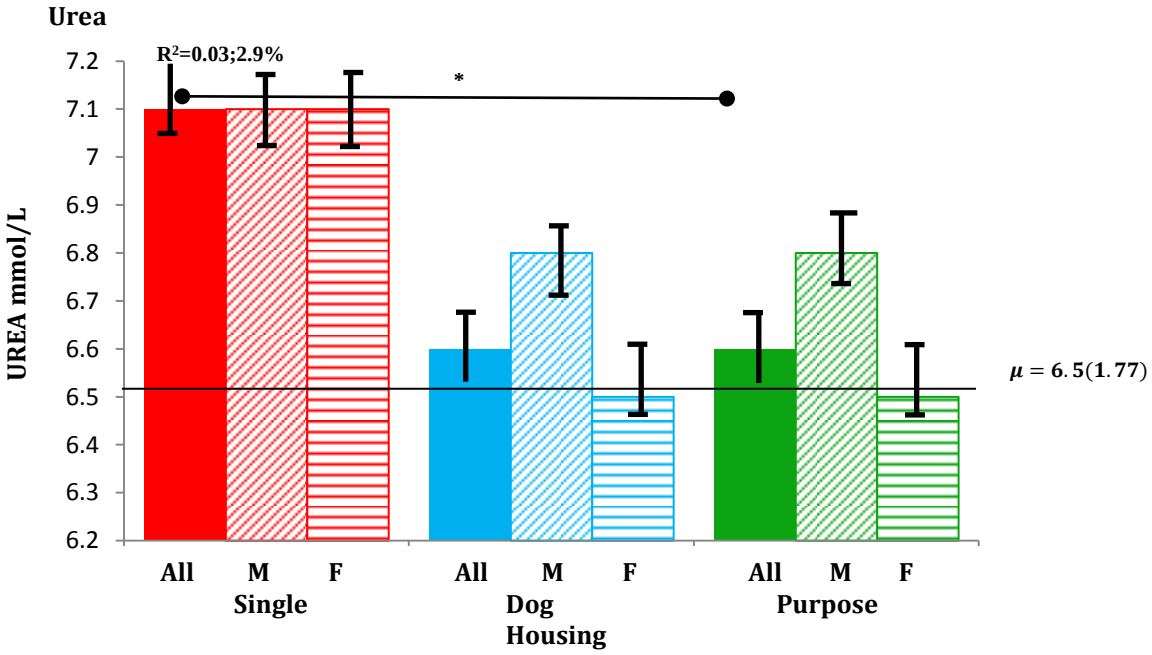
When age and acclimatisation time were controlled for, housing was found to have significant effects on levels of ALT in macaques, $F(2,698)=10.85$, $p<0.001$. Modified single housing resulted in highest ALT levels compared to purpose-built gang-caging, $t(698)=3.43$, $p<0.01$, $r=0.08$.



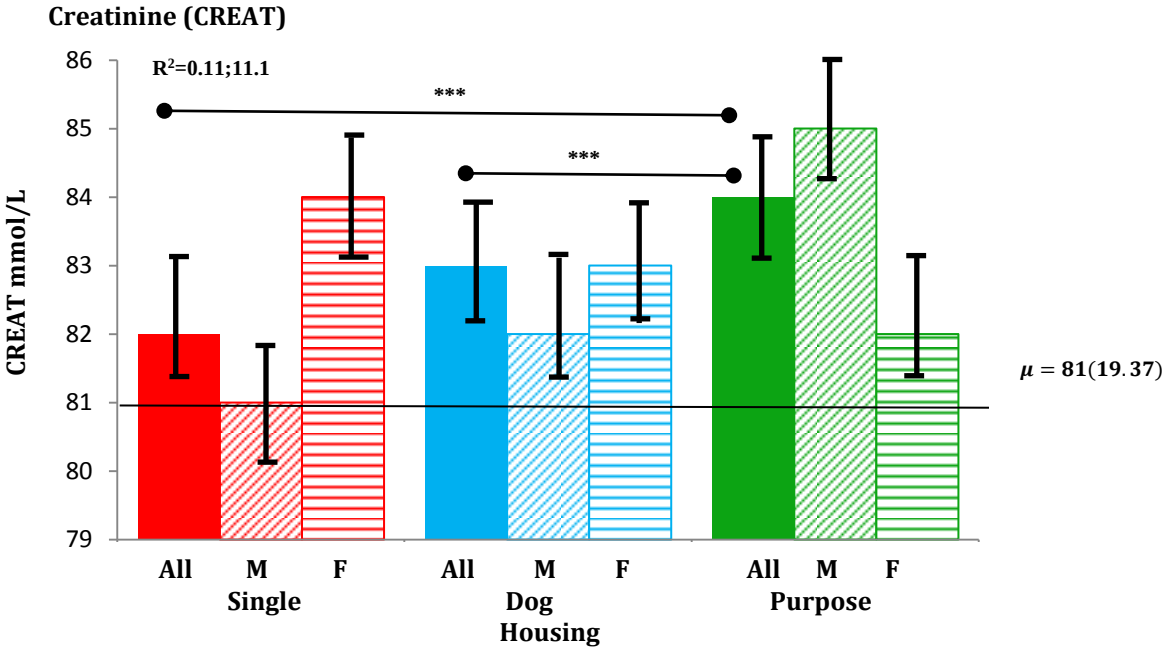
Housing condition had significant effects on GGT in macaques when controlled for age, sex and acclimatisaton time, $F(2,626)=15.43$, $p<0.05$. Macaques housed in the modified single condition had significantly higher GGT levels than macaques housed in purpose-built accommodation, $t(626)=1.66$, $p<0.05$. Similarly, GGT was elevated in macaques kept in modified dog accommodation, $t(626)=4.67$, $p<0.05$, $r=0.05$.



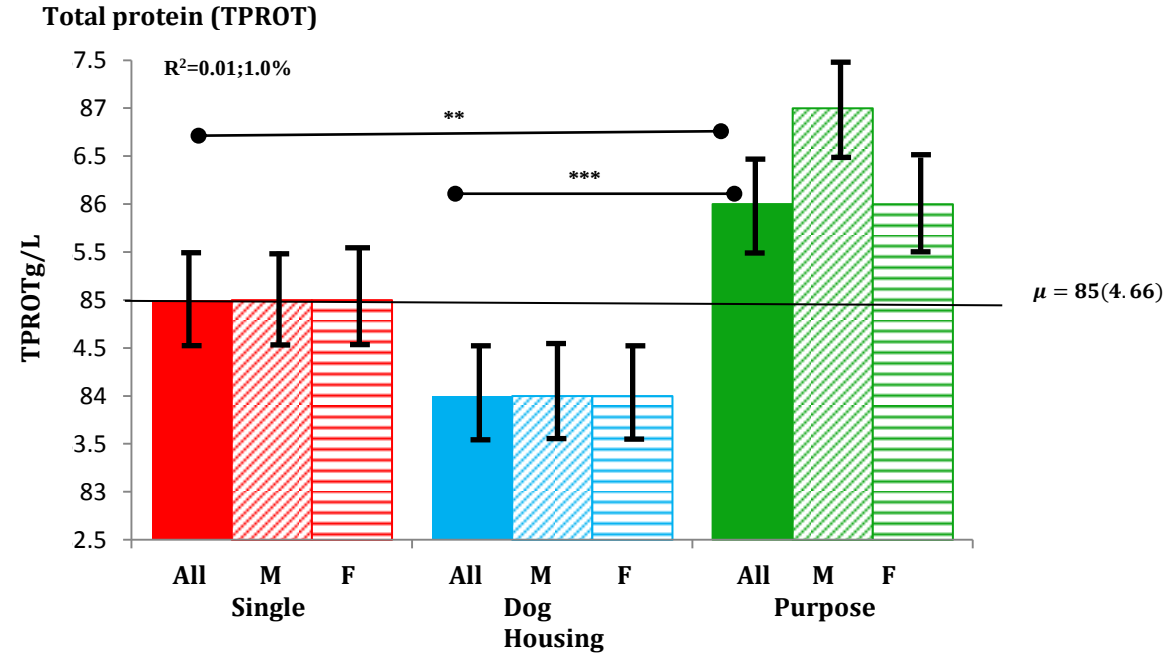
When controlled for acclimatisation time, age, number of sham doses and sex, housing was found to have a significant effect on baseline levels of ALP, $F(2,696)=2.96$, $p<0.05$. Macaques in modified dog pens had significantly higher ALP than those housed in purpose built gang-caging, $t(696)=2.35$, $p<0.02$, $r=0.40$.



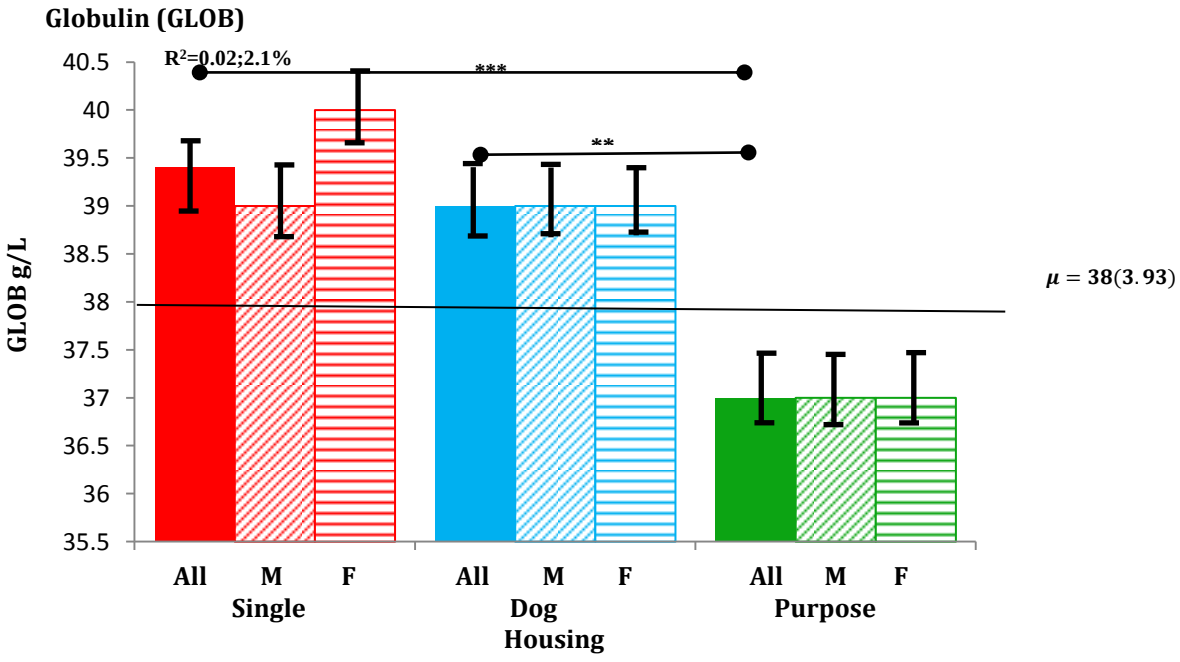
Urea concentrations were found to be significantly affected by macaques' housing conditions when controlled for sex, $F(2,818)=5.45$, $p<0.01$. Macaques kept in modified single cages had significantly higher urea concentrations than those in purpose-built housing, $t(818)=2.52$, $p<0.01$, $r=0.01$.



Creatinine was found to be significantly affected by housing when age and acclimatisation time were controlled for, $F(2, 698)=120.3$, $p<0.001$. Macaques kept in modified single, $t(698)=10.60$, $p<0.001$, and dog pens, $t(698)=14.47$, $p<0.001$, $r=0.57$ had lower CREAT levels than those in purpose-built gang-caging.

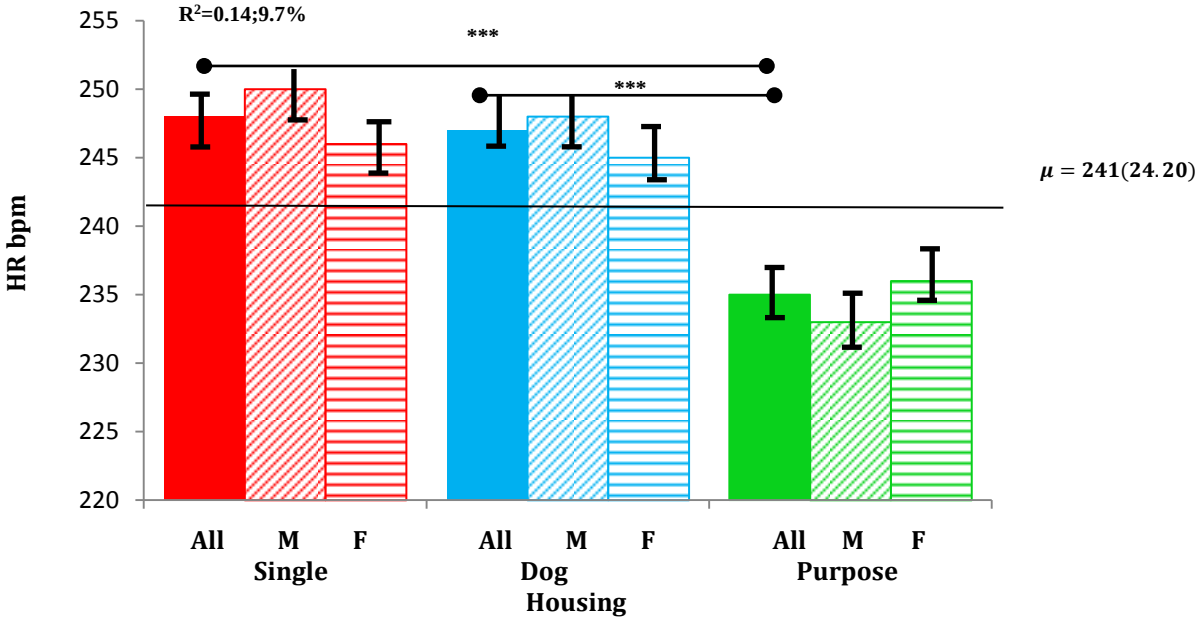


Total protein levels were significantly affected by housing condition, $F(2,698)=6.50$, $p<0.01$. Macaques in modified dog pens had the lowest levels compared to macaques kept in purpose-built pens, $t(698)=1.86$, $p<0.05$. Similarly lower TPROT levels were found in modified single caging $t(698)=3.56$, $p<0.001$, $r=0.25$.



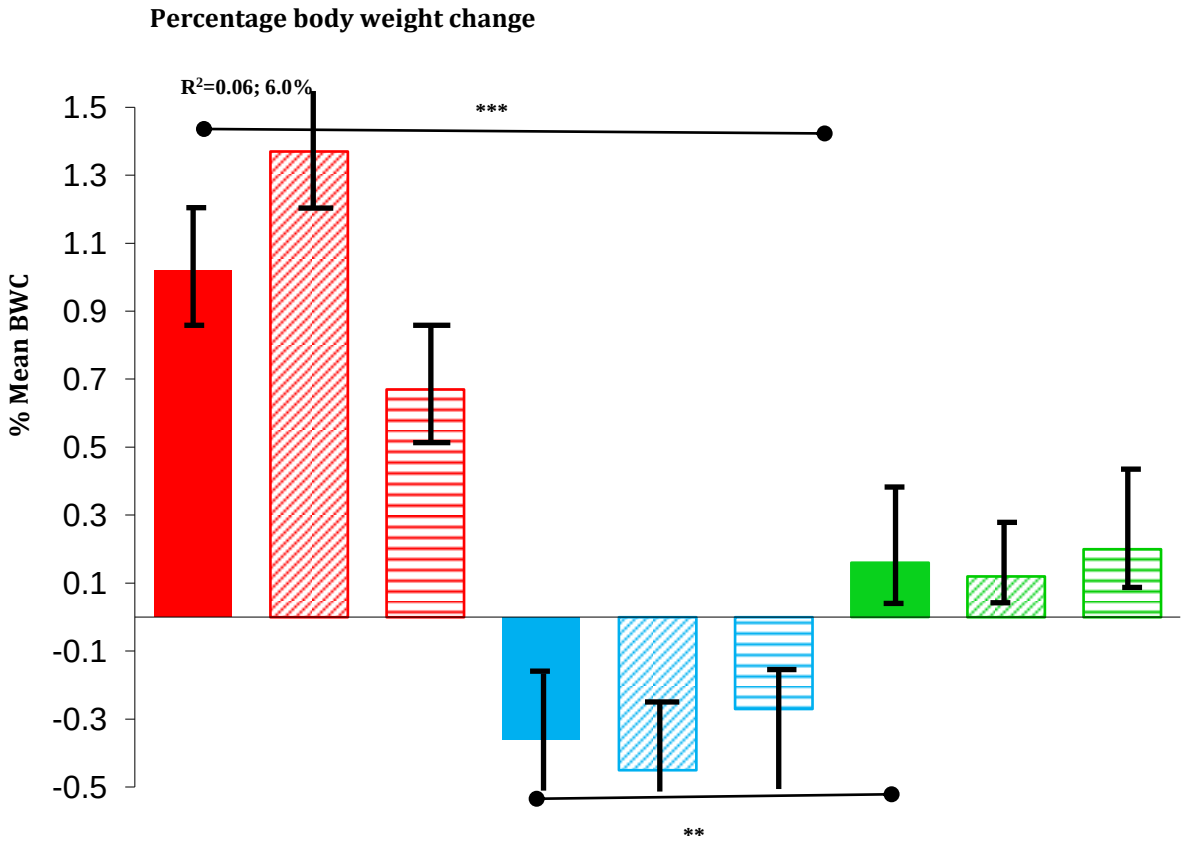
The level of circulating globulins was significantly affected by housing condition when controlled for the number of sham doses and age, $F(2,250)=8.26$, $p<0.001$. Both modified single and dog pens result in higher globulins compared to purpose-built gang accommodation; Modified single, $t(250)=3.81$, $p<0.001$, and dog, $t(250)=3.22$, $p<0.001$, $r=0.20$.

(c) Heart rate (HR)



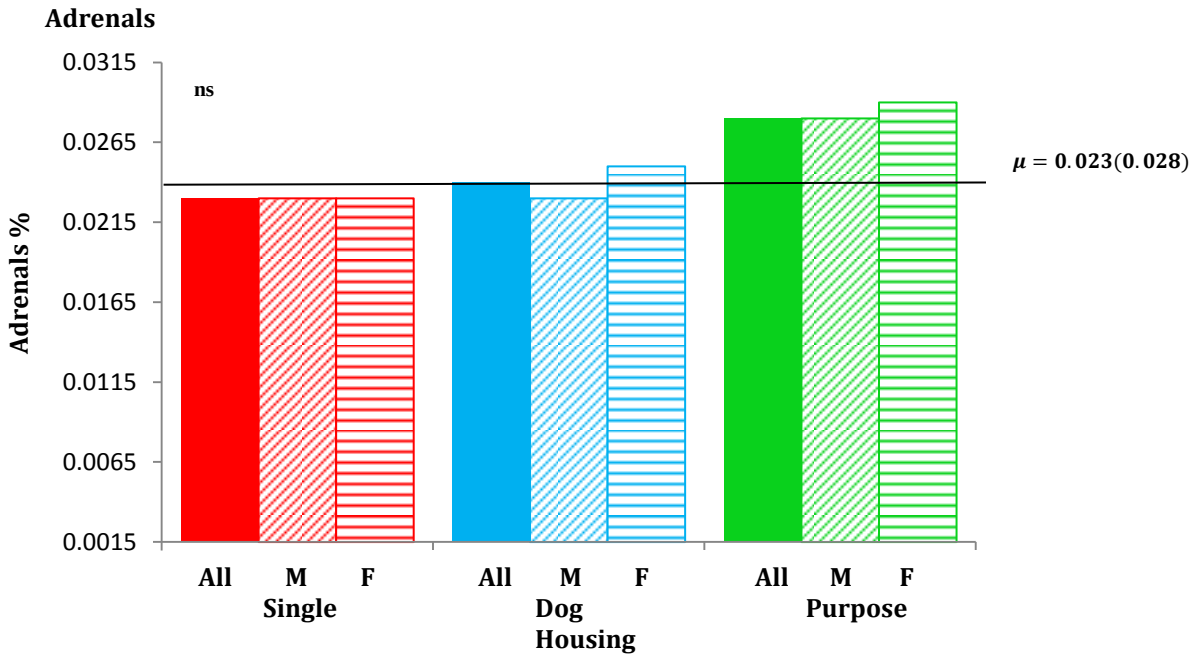
When controlled for acclimatisation time, housing was shown to significantly affect macaques' heart rate, $F(2,779)=27.70$, $p<0.001$. Macaques kept in purpose-built accommodation had significantly lower heart rates than those housed in either modified single, $t(779)=5.284$, $p<0.001$, or dog pens, $t(779)=6.46$, $p<0.001$, $r=0.10$.

(d) Percentage body weight change over 2 week pretreatment period (wk-2 to day 1of dose)

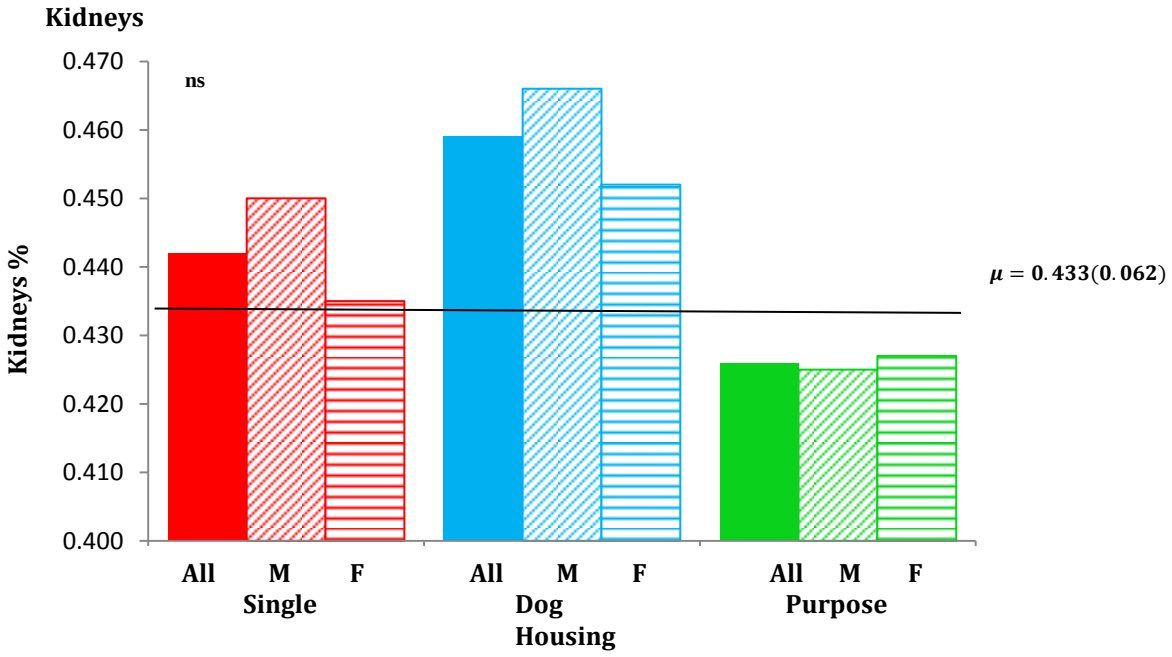


The mean percentage body weight change macaques experienced over the two week pretreatment period was significantly affected by housing condition when controlled for acclimatisation time and age, $F(2, 714)= 27.44$, $p<0.001$. Macaques in modified single cages gained significantly more weight than macaques housed in purpose-built accommodation. Those kept in modified dog pens lost weight in comparison to macaques in purpose-built housing; Modified single $t(714)=3.18$, $p<0.01$, and dog pens, $t(714)=-5.032$, $p<0.01$ $r= 0.05$.

(e) Organ:body weight percentages

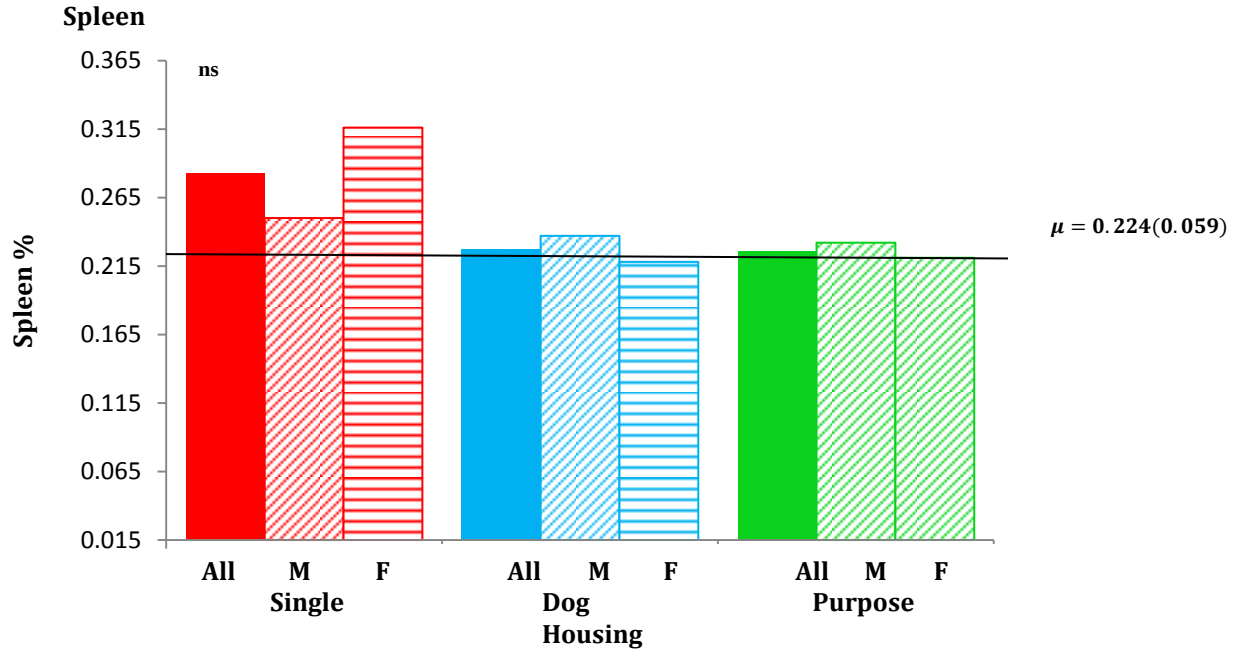


Housing was not found to significantly affect adrenal: body weight percentage; age, however, was found to have an effect (Table 4.3.4) and this may account for the higher adrenal weights seen with macaques housed in purpose-built accommodation, as these animals were older (Figure 4.3.1).

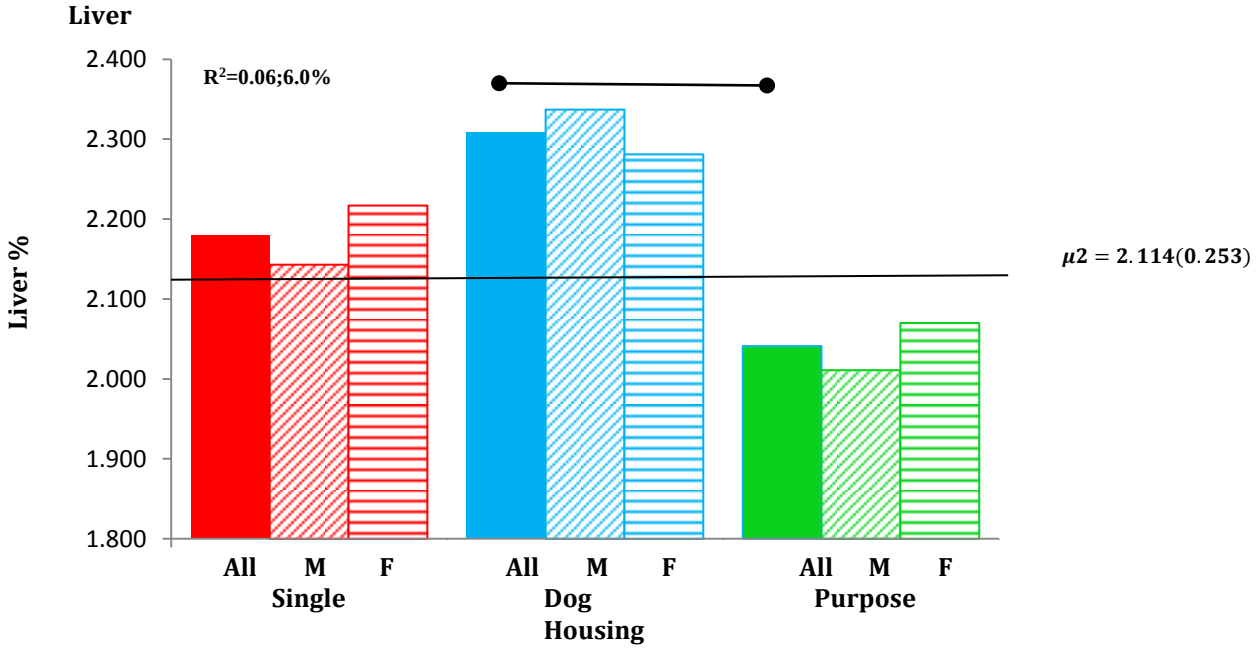


Age was found to be the only predictor variable that had an effect on kidney weight as percentage of body weight in macaques (Table 4.3.4); housing had no effect. Macaques in modified dog pens were the

youngest of the three housing conditions, whereas older animals were kept in purpose-built accommodation (Figure 4.3.1).



Spleen weights were not affected significantly by housing conditions, but by age and number of sham doses (Table 4.3.4). Sham dosing was only undertaken in one study included from the modified single housing condition.



Housing was found to significantly affect liver weights of macaques when controlled for age and number of sham doses, $F(2,144)= 12.88$, $p<0.001$. Liver weights of macaques housed in modified dog pens were significantly higher than macaques housed in purpose-built accommodation, $t(1444)=4.89$, $p<0.001$, $r=0.46$.

4.4 Discussion

Housing was found to have significant effects on the biological data of cynomolgus macaques (Figures 4.3.4a - e). However, as a result of the time frame over which changes in housing occurred, a number of other potentially confounding sources of variation were identified and these too were characterised in the selected population (Section 4.2.5; Table 4.3.4).

4.4.1 Characteristics of the population

The haematology and clinical chemistry reference intervals (Table 4.3.2) are derived from one of the largest reported cohorts of laboratory-housed cynomolgus macaques of Mauritian origin, and they are considered representative of healthy, juvenile and adolescent, male and female monkeys.

Clinical pathology reference intervals for this population were broadly in agreement with those published for laboratory-housed cynomolgus macaques (e.g. Peretta *et al* 1991; Terao 2005; Hall 2007; Bonfanti *et al* 2009). I found higher erythrocyte parameters (Hb; RBC; PCV) in haematological data compared to published studies, with the exception of data derived from macaques of known Mauritian origin (Bonfanti *et al* 2009). The reverse trend was found for PLAT; however, published data from Mauritian macaques were slightly lower. White blood cells (leukocytes) were more variable whilst my results overlap published values; they were most similar to those derived from Mauritian animals (Bonfanti *et al* 2009). Clinical chemistry analytes, AST and GGT were lower than most reported, including Mauritian animals. Absolute neutrophil values were also found to be higher than published levels for Mauritian macaques. Levels of ALT and ALP were highly variable; my data lie within the published ranges regardless of macaque origin.

Published data on heart rates from conscious (awake) restrained cynomolgus macaques are reviewed in Chapter 5. Pretreatment body weight change and organ: body weight data at end of life are sparse and therefore difficult to compare.

a) Age and sex effects

I found significant differences between male/female, and juvenile/young adult macaques. When examining the effects of a single variable (age or sex) my results agree only in part with the few published studies (Table 4.1.3). This is possibly a result of the large number of animals included in my cohort compared to others, and potentially my partitioning factors for age (juvenile vs. young adult) may be too narrow to detect some age-related changes (e.g. Section 4.2.5a.). Indeed, I did not include data from very young animals (<6mo) nor particularly old adults (> sexual maturity). There are, however, age*sex interactions accounting for variation in the reference data of macaques. For instance, Hb concentration in young adult males was higher than those of juvenile males, the reverse trend was observed for females. The picture becomes further complicated when all predictor variables (age, sex, acclimatisation time, sham dosing, duration of study and housing condition) were entered into the regression model. Age and sex accounted for variation in only 6 of the outcome variables (Hb, RBC, L, GGT, ALP, adrenals) compared to all others except three (WBC, pituitary and thyroid organs) when examined separately. This has implications for partitioning historic data on the basis of age and sex alone – a common practice when maintaining control databases for comparison, and sometimes aiding in interpretation of study data (James 1993; Hall 2007).

4.4.2 Variation in the data of the population

The baseline data from this population of cynomolgus macaques varied widely (reported standard deviations; ≤10% – ≥50% around the central mean) with parameters in the core battery. In part, this can be explained by variation in predictor variables (e.g. age, sex, acclimatisation time, study duration and housing condition etc.) across time. Despite quantifying these most obvious of predictors (see Section 4.1.5) into the model, a further 49-99% of the variation could not be explained. This is a wide range, specific for individual biological (outcome) parameters; for example only 1% of T Bili could be

explained by the predictors, whereas 61% of the variation in CREAT concentration could be accounted for. Individual biological parameters may be differentially sensitive to changes in the laboratory environment. The specific effects of any one or multiple changes in the study environment may be difficult to detect, often attributed to background variation (Chapter 2), but have the potential to impact on the reliability and repeatability of individual parameters. In turn this may confound our ability to determine whether drug-induced observations in the core battery recorded at the same time point are real or bad (Section 2.4; Figure 2.1; Hall 1997).

Being unable to identify all the influential factors (e.g. Figure 4.1.1) is a limitation when reviewing historic data, as only scant information on study conduct can be archived due to space limitations and confidential information pertaining to the study sponsor. However, procedural effects such as the sequence of venepuncture (e.g. Capitanio *et al* 1996), may have profound effects on circulating blood parameters of interest to toxicologists. Limitations with individual toxicology study design (Section 1.5.4; collection of samples in order of ascending dose group rather than randomized order, to prevent risk of cross-contamination) may increase variation as a result of procedural effects like those described by Capitanio and colleagues (1996). Nevertheless, without more detailed information regarding study procedures it is difficult to determine the degree to which these types of events influence the remaining variation in macaques' biological data, out with those relating to age, sex, acclimatisation time, study duration and housing condition. Furthermore, Table 4.3.1 highlights how difficult it is for toxicological establishments to standardise animal-related variables on study; this is in stark contrast to many nonrodent studies (Chapter 2). Despite difficulties in interpreting the data there are some interesting trends alongside transition in housing conditions (Section 4.4.2) that require cautious interpretation.

4.4.3 Effect of housing and husbandry on macaques' biological data

Housing was found toexert differential effects on the biological data of cynomolgus macaques in all but two clinical pathology analytes (M and TBili), and in only one organ parameter: liver:body weight ratio. The amount of variation attributable to housing in each case was variable, but not large (0.5-11.1%);

the largest effect was observed on CREAT (11.1%) levels and heart rate (9.7%). It should also be noted that the data across housing conditions for all biological parameters were within published ranges (Section 4.4.1) and historic control data held by the CASE sponsor (unpublished data). Examining the link between housing and biological data is further complicated by the multiple physiological functions of some parameters (Appendix 1.1). For example, CREAT was higher in macaques housed in purpose-built rather than modified dog or single cages (Figure 4.3.4b). Creatinine (CREAT) is produced in muscle, particularly skeletal, but it also indicates renal function (Loeb 1989; Hall 2007; Appendix 1.1). Assuming only healthy animals were included onto study, changes in renal function are unlikely to account for the higher levels of CREAT observed in animals housed in purpose-built accommodation. Although not reported by other authors to increase with age (Table 4.13), CREAT levels may be higher in older (Figure 4.3.1), heavier animals (unreported data) in the purpose-built housing condition owing to their increased skeletal muscle mass (Hall 2007).

Nevertheless when controlling for age, CREAT (Figure 4.3.4b) was higher in animals in purpose-built accommodation, possibly in relation to being handled for sham dosing. Animals that struggle during restraint may have elevated CREAT levels as a result of secondary iatrogenic muscle injury (Hall 2007), and animals in purpose built gang-housing experienced more sham dosing than in any other housing condition (Figure 4.3.3) - a procedure macaques don't readily or ever habituate to (Fante *et al* 2012). This pattern of CREAT concentration is not mirrored by ALT and AST levels (Figure 4.3.4b), other useful markers of tissue damage, although they are less reflective than CREAT of skeletal muscle damage and more so of liver and cardiac tissue breakdown (Appendix 1.1). Although CREAT concentrations across all three housing conditions were not outside the published normative ranges, if CREAT is elevated at baseline it may make it difficult to quantify adverse changes (Chapter 2) in response to chronic dosing. However, given the non- specificity of CREAT, any changes would be interpreted in the context of changes with other core battery measures (e.g. blood urea concentrations, ALT and ASP levels; Hall 2007).

Heart rate was lower in animals housed in purpose-built accommodation compared to single and modified dog pens. Heart rate is sensitive to psychological and physical stress experienced during handling, restraint and care staff contact (reviewed in Chapter 5). Indeed changes in restraint, habituation to ECG recording procedure, and enhanced socialisation with care staff have been found to reduce macaques' heart rate (Chapter 5). The characteristics of single housing can prohibit formation of positive human-macaque interactions (Section 4.1.4b; Table 4.2.3). The small cage dimensions and tiered arrangement, prevents macaques from having control over human approach. For example, they're not able to sit off the pen floor or above the head of human care staff, nor can they perform a vertical flight response to get away from staff when they feel frightened. Furthermore, hand capture techniques were used to remove macaques from their pens, bringing staff and macaques into direct conflict with one another. Hand capture requires care staff to wear personal protective clothing (e.g. visor and gauntlets) and to approach macaques face-on, before manually removing macaques from the pen; a procedure macaques are likely to find threatening.

However, given the physical similarity between modified dog and purpose-built accommodation it is surprising to observe a reduction in heart rate, which likely results from design features in purpose-built accommodation that promote a more positive relationship with care staff during their daily activities (Table 4.2.3; Chapter 5). For example, hand capture was employed in modified dog pens, with care staff entering the pen to catch macaques, increasing the opportunity for conflict. Furthermore, additional grids placed over the outside of existing dog pens prevented care staff from hand feeding macaques during routine husbandry. Moreover, suspended perches were sited at the back of the pen, further reducing the incentive for macaques to voluntarily approach care staff and watch human activity. Conversely, in purpose-built accommodation both these design features had been altered. With the addition of a 'hatch' at the front of the pen, macaques could run voluntarily into transport boxes, avoiding physical contact with care staff during capture. Further, multiple suspended perches at the front, side and back of the pen, and wide bars at the front of the pen provided macaques with a good view of human activity and protected contact, thereby facilitating hand feeding during routine husbandry events.

Given that sham dosing was mostly performed in the later housing condition, one might therefore predict because of the aversive nature of handling associated with care staff on the days preceding recording ECGs that macaques would have elevated heart rates. The stress-mitigating effect of socialisation with care staff on a daily basis seems to have a greater impact on heart rate than other Refinements (e.g. acclimatisation time, habituation to tube restraint; Chapter 5). Building a positive relationship with care staff during normal husbandry events may buffer macaques against negative experience of aversive handling associated with sham dosing (Chapter 5). Despite a reduction in heart rates with improved housing, they are high (e.g. manual restraint: mean 235 bpm; tube restraint: mean 225 bpm, Chapter 5; freely moving telemetered: mean 159 bpm, Bass *et al* 2009) and undesirable for accurately determining all the waveforms in an ECG trace when assessing the cardiotoxic effects of novel pharmaceuticals (e.g. Kelly 2009; Chapter 5; Appendix 1.2).

Of the immunological parameters in the core battery (e.g. WBC, N and L counts; Appendix 1.1), purpose-built group housing produced higher WBC, N and lowest L counts; a pattern typical of alarm reaction Leukogram seen in response to handling and restraint (Section 4.1.5ei). Given that handling for sham dosing is nearly always carried out in the later housing condition and rarely performed on animals housed in single or modified dog pens (Figure 4.3.3), perhaps this Leukogram pattern is not a reflection of housing condition but rather a change in standard operating procedure (Section 4.2.5).

There are some effects that may not be directly attributable to housing, as the rationale for differences are at odds with their known physiological function. For example, lower levels of ALP observed in older animals housed in purpose-built accommodation (Figure 4.3.4b). Alkaline phosphatase is a measurement of joint enzyme activity and is higher in growing animals (Appendix 1.1); the rate of growth in younger animals housed in modified single and dog accommodation may account for higher levels. This potential growth pattern is in part reflected by differences in patterns of body weight change during the two-week pretreatment period across housing conditions (Figure 4.3.4d); older macaques in purpose-built accommodation gain less weight than younger animals in the single housing condition. However, age and ALP levels in juvenile macaques housed in modified dog cages were

similar to their singly housed counterparts, yet they lost weight during pretreatment. This is somewhat surprising given that they also spent longer in the unit acclimatising to local conditions and we assume they were more stable before onset of study (Section 4.1.5d and Chapter 2 for review). The difference may be attributable to the negative effects of sham dosing. However, sham dosing was more common in purpose-built gang-caging, where a small weight gain was observed. Body weight data in Chapter 5 show that animals experiencing enhanced socialisation with care staff had more stable weight gain than controls during the pretreatment period, indicating that socialisation may buffer macaques against stressful handling procedures.

4.5 Conclusion

The degree to which welfare-positive changes in housing affected macaques' biological data varied considerably. Untangling the effects of sex, age, acclimatisation time, sham dosing and study duration alongside the changes related to housing proved quite challenging. Handling and restraint associated with sham dosing prior to day 1 of dose has negative consequences for welfare and baseline biological data. However the design of purpose-built gang-caging may reduce conflict between care staff and macaques, as it gives animals more control over their environment and fosters positive-human interaction during routine husbandry, which appears to buffer macaques against the aversive nature of sham dosing. The specific effects of socialisation with care-staff for macaque welfare and quality of scientific output are examined in Chapter 5.

5

The effects of enhanced socialisation with care staff on macaque welfare and toxicological measures

"….refinement might be regarded as an art or an ability to improvise."

Russell & Burch (1959), p 134.

"Refinement will inevitably increase efficacy, and may incidentally entail reduction as well. If not grossly more expansive in itself (in terms of apparatus and skilled staff), it is always more efficient."

Russell & Burch (1959), p 156.

"The Kra is not easily domesticated."

Raffles (1821), p 247.

Abstract

*Human care staff are critical for good welfare and good quality scientific outcomes in the laboratory. They are a necessary feature in the lives of all non-human primates used for research and testing. Close contact is regularly required for the performance of husbandry and regulated procedures, yet cynomolgus macaques (*Macaca fascicularis*) are not domesticated and may perceive humans as a threat, reacting fearfully or aggressively when in close proximity. Positive interactions between care staff and macaques are known to improve their health and welfare and their ability to cope with stress in response to husbandry and scientific events. Indeed ensuring care staff have a positive influence on welfare has been singled out as the most important Refinement that can be applied in the laboratory (e.g. Petto* et al *1992), and it is recommended as a priority in animal care guidelines. The process of habituating and socialising primates to the behaviour, sight, sound and smell of humans, may help to avoid or reduce fear responses and facilitate handling for routine husbandry and regulated procedures. Furthermore, cardiovascular parameters (e.g. heart rate and blood pressure), which form essential components of the core battery used to evaluate toxicity during safety and efficacy testing, are particularly sensitive to psychological stress that accompanies fearful experiences. Fear is not conducive to happiness, an inherent feature of good welfare (Poole 1997), and may confound our ability to interpret cardiovascular data, introducing undesirable variation into heart rate and blood pressure measurements used to assess risk prior to human exposure. In this final experimental Chapter I aim to explicitly examine the link between Refinement, animal welfare and quality of scientific outcomes in cynomolgus macaques used for regulatory toxicology. Socialisation with human care staff was found to enhance welfare (e.g. reduced fear responses), facilitate procedures and increase the sensitivity and reliability of heart rate and blood pressure parameters, achieved through lower baseline values, with less between-animal variation. This study provides quantitative data demonstrating how Refinement improves the quality of toxicological output through better welfare.*

5.1 Introduction

In Chapter 3, I highlighted the critical role of human care staff for animal welfare; which relies on their ability and skill for monitoring, evaluating and promoting positive opportunities to enhance the lives of primates in laboratories. However their influence extends beyond monitoring and evaluating the animals and their environment; the very nature of human-animal interactions affects every aspect of the use of primates in research (Rennie & Buchanan-Smith 2006a). Human care staff can be a potent, salient environmental stimulus, evoking positive or negative reactions in animals (reviewed in Rennie & Buchanan-Smith 2006a; Manciocco *et al* 2009). They are rarely benign, altering their behavioural and physiological parameters (Line *et al* 1989; 1991; Flow & Jaques 1997; Reinhardt 2004). The extent of these human influential factors often goes unnoticed yet they have the capacity to introduce undesirable variation into experimental results and confound research outcomes (Capitanio *et al* 1996; Flow & Jaques 1997; Reinhardt & Reinhardt 2000; Reinhardt 2004; Chapter 2).

Close contact is indeed a necessity of good animal care programmes (Chapter 3), and it has been recognised that care staff who work closely with animals are often the first to notice changes in response to the negative impact of procedures (Wolfe 2002). Furthermore their ability to recognise the significance of subtle alterations in behavioural responses is essential for evaluating methods to improve welfare (Bayne 2002; Rennie & Buchanan-Smith 2006a). Nonetheless the presence of humans is as important as it is controversial in the lives of all captive animals (Manciocco *et al* 2009). The behavioural, physiological and biochemical effects of human interaction have been most comprehensively characterised in domesticated animals housed in farming systems (Hemsworth *et al* 1993; Breuer *et al* 2000). However, although captive bred, primates used in research are not domesticated and close contact with humans has the potential to be stressful especially where they cannot control or predict it (Scientific Committee on Animal Health & Welfare 2002; Waitt & Buchanan-Smith 2001; Waitt *et al* 2003; Bassett & Buchanan-Smith 2007; JWGR 2009).

For example, in macaques, the mere presence of care staff or an experimenter results in elevated heart rates and alters behavioural responses (Malinow *et al* 1974; Line *et al* 1989; 1991; Maloney *et al* 2007). Indeed these characteristic responses have been used in a controlled manner in

experimental paradigms to investigate the physiological stress response in monkeys (e.g. Clarke *et al* 1993; 1995; Bowers *et al* 1998; Kaplan *et al* 2009; Shively *et al* 2009). Exposure to the presence of an unfamiliar person, threat of capture and manual restraint by care staff form the basis of 'stress' tests (e.g. Clarke & Mason 1988; Clarke *et al* 1993; 1995) because they produce predictable effects on the autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal axis (HPA) (Chapter 3). Furthermore, they are accompanied by characteristic fearful or aggressive behavioural responses (Clarke *et al* 1993; 1995; Manuck *et al* 2009; Shively *et al* 2009). These responses may be triggered inadvertently by humans caring for primates in laboratories particularly if animals have not had opportunities to build positive relationships with care staff (e.g. newly acquired macaques) or staff become associated with negative events (e.g. alarming, painful or distressing procedures; Rennie & Buchanan-Smith 2006a). Indeed, such fear responses may introduce unwanted variation into experimental variables (Buchanan-Smith 2006a), especially those modulated by activation of the ANS and HPA such as cardiovascular, immune, haematological and clinical chemistry parameters (Chapter 3). These physiological parameters are critical components of the core battery used to evaluate toxicity in animals prior to human exposure (Chapter 1). It is vital therefore that these types of responses are avoided, and this can be achieved by promoting positive interaction between human care staff and macaques (Rennie & Buchanan-Smith 2006a).

Sympathetic, positive interaction between care staff and primates, in a manner that is meaningful to primates has been found to enhance their welfare (reviewed below; JWGR 2009). For example human interaction associated with training programmes has been used to improve nearly every aspect of the lives of primates housed in laboratories (e.g. during husbandry, scientific procedures, group formation, feeding, environmental enrichment and behavioural management of abnormal behaviours; reviewed in Prescott *et al* 2005; Rennie & Buchannan-Smith 2006a; Prescott & Buchanan-Smith 2007). Even less structured approaches to interaction, those that happen spontaneously during daily contact, can affect the behaviour of primates and their capacity to cope with stress (Lambeth *et al* 1997; Baker 2004; Baker & Springer 2006; Clay *et al* 2009; Manciocco *et al* 2009). Habituation and socialisation (Table 5.1.1) of primates to the presence (sight, sound, smell) and behaviour of humans is recommended to avoid or reduce fear responses (JWGR 2009)

and can also facilitate handling and restraint, reducing the need for personal protective equipment and even sedation for procedures (Heath 1989; Reinhardt *et al* 1995a; McKinley *et al* 2003).

Table 5.1.1 Definition of learning processes for Refinement of care staff contact.

Term	Definition
Habituation	The waning of a response as a result of repeated stimulation, but not fatigue.
Socialisation	The process by which primates learn how to successfully interact with members of their own and other species (e.g. humans).
Desensitisation	Systematically pairing positive reward directly with an uncomfortable or aversive experience or stimulus in order to reduce any associated fear or anxiety response.
Training	The shaping of the behaviour of a primate so that it actively responds in a way that is desired by the trainer.
Positive reinforcement	The process of increasing the frequency of a behaviour by introducing something positive on its performance.

Prescott *et al* (2005); Prescott & Buchanan-Smith (2007).

5.1.1 Influence of humans in laboratories

The perceptual capabilities and cognitive capacity of primates determine how they perceive and respond to environmental stimuli including humans (Prescott 2006; JWGR 2009). Primates can readily recognise individual humans (Sands & Wright 1982). They show preferences for those with whom they have had positive interactions (Bloomsmith *et al* 1997) and exhibit signs of fear and aggression towards humans associated with negative experiences (McKinley 2004). They can distinguish between quiet, calm and loud, forceful human voices which may alarm them or be used to positively reinforce behaviours for cooperation during training (JWGR 2009).

Nevertheless routine monitoring and observation of macaques by familiar personnel may result in persistent stress responses (e.g. Malinow *et al* 1974; Manuck *et al* 1983; Line *et al* 1989; 1991). For example, Kim and Han (2006) found distinct patterns of activity in macaques housed in the laboratory to be associated with husbandry events. Indeed, both Malinow *et al* (1974) and Line *et al* (1991) found similar patterns of activity were accompanied by changes in heart rates in rhesus macaques (*Macaca mulatta*). Malinow *et al* (1974) recorded peak heart rates during feeding and room cleaning. Similarly, Line *et al* (1991) found heart rates elevated with the mildly aversive events associated with room cleaning, whilst a second peak occurred at afternoon feeding, before heart rates gradually declined over the rest of the day until lights out. Nonetheless, given that these events were daily occurrences, the monkeys continued to react to them. Furthermore, the authors found heart rates remained elevated for several hours after cage changing and tuberculin (TB) testing, and despite repeated exposure to these events their more aversive nature meant monkeys were slower to recover baseline heart rates and did not habituate to them (Line *et al* 1991).

Clearly, in the laboratory it is not possible or even desirable to eliminate or reduce the frequency of these events, as they are essential for maintaining animals in good health. However, as described above, a passive approach to habituation through repeated exposure to these regular occurrences was not sufficient to reduce their negative impact on macaques. This necessitates a more thoughtful and active method which aims to alter animals' reactions to fear-provoking stimuli through the principles of positive reinforcement training (PRT) and systematic desensitisation (Clay *et al* 2009; Table 5.1.1). Such positive approaches when paired with fear evoking stimuli encountered on a daily basis may reduce some of the harmful effects on animals. For example, Clay *et al* (2009) found that desensitising singly housed rhesus macaques to events they had previously found fearful, through a process of pairing high value food treats with systematic presentations of the fear inducing stimuli had positive effects on macaques' behaviour. They observed a significant reduction in stress-related behaviours (e.g. yawning, body-shake, self-scratching), cringing behaviours in general, and cringing directed towards human care staff.

Furthermore, positive human interaction has been proposed as a type of environmental enrichment for laboratory-housed primates (Bloomsmith *et al* 1999; Baker 2003). For example, Baker (2003) found singly housed macaques displaying self-injurious behaviours when exposed to more positive human interaction showed an increase in the use of enrichment items and higher levels of self-grooming. Furthermore in chimpanzees (*Pan troglodytes*), Baker (2004) found that providing additional positive human interaction resulted in a reduction of abnormal behaviour, and the chimpanzees appeared less tense and reactive overall, engaging in higher levels of affiliative behaviours. A similar finding was reported for macaques by Bayne *et al* (1993), where human interaction facilitated through treat provisioning reduced the incidence of abnormal behaviours in singly housed rhesus macaques.

The effects of human interaction as enrichment for great apes found both positive reinforcement techniques (PRT) and less structured interaction conferred different benefits to chimpanzees (Bloomsmith *et al* 1999). Whilst PRT appeared to benefit social behaviour more broadly, less structured interaction ameliorated stereotypic and anxiety related behaviour (Bloomsmith *et al* 1999). Similarly, in common marmosets (*Callithrix jacchus*) an unstructured approach based

around 20 min/day extra interaction with a familiar carer over five weeks resulted in significant alterations in behaviour (Manciocco *et al* 2009). Play, grooming and affiliative behaviours increased whereas self-scratching and emission of phee (alarm) calls decreased; the authors also observed a decreasing trend in time spent locomoting and resting (Manciocco *et al* 2009).

Moreover, Markowitz and Line (1989) reported that in the quiet presence of humans that occasionally provided treats, but mainly watched behaviour, there was a considerable reduction in abnormal behaviour associated with human care staff entering the colony room. Indeed, Waitt *et al* (2002) found that stump-tailed macaques (*Macaca arctoides*) that showed affiliative behaviour towards care staff exhibited less abnormal behaviour than those which avoided or were aggressive towards care staff. Given the body of evidence above, it is not surprising that human interaction is recommended to enhance captive environments (Markowitz & Spinelli 1986; Wolfe 1987; 1996; Novak & Drewson 1989; Bennett 1990; Mahoney 1992; Bloomsmith *et al* 1997; 1999; Baker 2003; 2004; Clay *et al* 2009; Manciocco *et al* 2009) and enables primates to cope better with routine events (Lambeth *et al* 1997; Rennie & Buchanan-Smith 2006a).

5.1.2 Refinement of human contact in laboratories

Positive human interaction is also recommended as a priority in animal care guidelines (e.g. Home Office 1989; NRC 1996; 2011; Scientific Committee on Animal Health & Welfare 2002; NC3Rs 2006; Council of Europe 2007; International Primatological Society 2007; JWGR 2009; EU 2010; Laule 2010) and singled out as the most important Refinement that can be applied in the laboratory environment (Petto *et al* 1992). Useful strategies for habituating and socialising primates to humans have been highlighted above (see JWGR 2009) and the key to their effectiveness lies in the formal implementation of programmes based upon the principles of positive reinforcement (Table 5.1.1), that involve all staff who come into contact with the animals and that continue throughout the animal's life (Prescott *et al* 2005; Rennie & Buchanan-Smith 2006c; Prescott & Buchanan-Smith 2007; JWGR 2009).

There is both an ethical and scientific need to Refine human contact in laboratories to obviate the negative effects of fear and stress responses with uncontrollable and or unpredictable human

interaction (Rennie & Buchanan-Smith 2006a; Bassett & Buchanan-Smith 2007; JWGR 2009). Given that stress, through activation of the autonomic nervous and endocrine systems has potentially confounding effects on some of the physiological parameters measured by toxicologists (Chapter 2 & 3), I review the importance of cardiovascular parameters in the safety and efficacy screening of new drugs below.

5.1.3 Importance of cardiovascular measures in toxicology

Current regulatory guidelines for safety assessment of all new chemical entities require evaluation of toxic effects on cardiac function in experimental animals prior to human exposure (Chapter 1; ICH guidelines S7A; S7B; ICH 2005; Chaves *et al* 2006). Electrocardiographs (ECG) coupled with blood pressure measures recorded at multiple time points are essential in evaluating the potential toxic effects of compounds on the heart (Hamlin *et al* 2004). In order to accurately quantify the magnitude and direction of any induced changes it is critical that baseline recordings are free from confounding factors and undesirable variation (Chapter 2; Gauvin *et al* 2009).

To put this in the context of the human patient, even small systemic arterial pressure elevations are recognised as a risk factor for mortality (Kannel 2000). For example, 2 mmHg change in blood pressure translates into a 10% change in stroke risk and 7% change in risk of mortality from ischaemic heart disease (Lewington *et al* 2002). Furthermore, wave forms in the ECG recording give valuable information on the underlying electrophysiological function (e.g. repolarization and depolarization of cardiac muscle cells) that result in the heart pumping blood around the body (Appendix 1.2). Perhaps most importantly, an alteration of cardiac function such as lengthening of ventricular repolarization (Appendix 1.2) is known to be a risk factor for the development of Torsades de Pointes, a potentially fatal ventricular tachycardia in humans (Roche *et al* 2005; Champeroux *et al* 2009). This effect represents a significant public health issue in the development of new drugs (Friedrichs *et al* 2005), not least because this type of electrophysiological alteration has been induced by a wide range of human pharmaceuticals, not just drugs intended for managing cardiovascular conditions (Friedrichs *et al* 2005; Bass *et al* 2009; Champeroux *et al* 2009).

There are several levels of electrophysiological evaluation that are generally used to assess ventricular repolarization (e.g. Bass *et al* 2004; Kinter *et al* 2004) including evaluation of ECG waveforms (Friedrichs *et al* 2005). Indices of prolonged ventricular repolarization in animal models used in toxicology include increases in QT or rate-corrected QTc intervals derived from the ECG traces (Hamlin 2006; Champeroux *et al* 2009; Appendix 1.2). A recent ICH guidance document for clinical studies on cardiac repolarization (e.g. E14) highlighted the threshold at which changes in QTc intervals (e.g. small range: 5 – 10 ms) would be considered significant for risk factors associated with proarrhythmia in humans (Holzgrefe *et al* 2007). Such small changes serve as important biomarkers for risk factors associated malignant arrhythmias (Friedrichs *et al* 2005; Hamlin 2006; Champeroux *et al* 2009), and these observations highlight the importance of sensitive evaluation methodologies for human safety in drug development (Authier *et al* 2008).

Psychological stressers such as novelty and restraint have pronounced effects on the ANS – a system that regulates heart rate and blood pressure (Chapter 3; Appendix 1.2). Furthermore, heart rate and QT interval duration are inextricably linked (see below), but more recently it has become apparent that QT interval is substantially modulated by autonomic tone (Champeroux *et al* 2010) and it too is potentially affected by psychological stress (Fossa 2008).

Whilst advanced technologies for optimizing the acquisition, analysis and monitoring of cardiac function *in vivo,* in freely moving animals, have greatly improved the ability to detect drugs that are cardiotoxic (e.g. in conscious telemetered animals; Holzgrefe *et al* 2007; Authier *et al* 2008; Chaves *et al* 2006; Bass *et al* 2009; Guth *et al* 2009; Champeroux *et al* 2010), they are often costly and impractical for use in mainstream regulatory toxicology (Chaves *et al* 2006).

Nonetheless, without Refinement, restraint stress confounds the ability of toxicologists to detect drug-induced changes in cardiovascular parameters which are important biomarkers for human safety. For example, Bass *et al* (2009) compared ECG traces collected from conscious, telemetered, freely moving and restrained macaques at baseline and after repeated treatment with a drug candidate known to affect cardiac ventricular repolarization. They found baseline heart rates in restrained animals were higher compared to telemetered animals (e.g. restraint: 242 ± 17.2 bpm;

telemetered: 159 ± 22.1 bpm), which inevitably reduced sensitivity to the drug during testing through the constraints of ceiling effects. Furthermore, the sensitivity of detecting proarrhythmia-accelerating effects of the drug were reduced by a factor of at least 2 (e.g. restrained: QTc prolongation 29 ± 12 ms; unrestrained: 65 ± 23 ms). Not only was the magnitude of the drug-induced effect on QTc interval reduced, but alterations in T wave morphology (Appendix 1.2) were only evident after dose in unrestrained macaques (Bass *et al* 2009). Similarly, in marmosets (*Callithrix jacchus*), heart rates recorded from conscious, freely moving, telemetered animals were a third less than those recorded from conscious, restrained animals (e.g. restrained: 348 bpm; telemetered: 230 bpm; Schnell & Wood 1995). Furthermore, when administered with a drug known to induce tachycardia, the magnitude and duration of its effects could only be determined in telemetered marmosets; no drug effects were detected in restrained animals owing to high baseline heart rates (Schnell & Wood 1993).

The ceiling effect of restraint therefore prevented accurate quantification of the cardiotoxic effects of the drug candidate. The drawbacks of restraining macaques for characterising drug effects on cardiovascular parameters are recognised by the CASE sponsor (Kelly 2009). Indeed, they have dedicated time and resources to optimizing ECG and blood pressure recording through the use of digitized recording platforms, high definition oscillometry, implanted and jacketed external telemetry. Furthermore, in general toxicology studies the use of digital ECG recordings necessitated Refinement of the restraint method, moving away from manual restraint by technician to tube restraint (Kelly 2009). The change in restraint resulted in consistently lower mean heart rates (Figure 5.3.3 j) in macaques, and this study piggy backs on this positive change by further Refining care staff-macaque relationships in newly acquired animals.

5.1.4 Aims of the Chapter

Given the important influence of humans over the lives of laboratory-housed macaques, a high priority for Refinement is the macaque – care staff relationship. Positive human interaction has been found to have beneficial effects on the health and welfare of macaques and their ability to cope with routine husbandry and scientific procedures. The effect of this important Refinement was chosen to examine the link between welfare and quality of scientific outcomes in cynomolgus

macaques used for toxicology. Using the frameworks to assess quality of toxicological outputs given in Chapter 2 and assessment of welfare developed in Chapter 3, the aim of this Chapter was to evaluate the effects of enhanced socialisation with care staff on macaque welfare and toxicological measures. Specifically, do we improve welfare and quality of scientific outcomes with socialisation? Do we observe measurable improvements in heart rates and blood pressure, recording lower baseline levels, and do we enhance the reliability of these parameters by reducing variation between animals?

5.2 Methods

5.2.1 Animals, housing and husbandry

Animals

Two cohorts (termed: 'Control(s)' and 'Enhanced') of juvenile and young adult (4.2.5a) male and female cynomolgus macaques, purpose-bred in Mauritius were observed from arrival to start of study during acclimatisation and baseline acquisition of core battery cardiovascular measures (e.g. ECG: heart rate; HDO: systolic, diastolic and mean arterial pressures; Table 5.2.1). Animals arrived in the same batch, originated from the same breeder and were naïve, not used in any other toxicological investigations. Cohorts were matched up to the end of week 5 on the unit when 'Control' macaques were assigned to study and pretreatment procedures were performed in week 6. Unfortunately, the 'Enhanced' group were delayed on to study and then diverted onto a male-only study requiring a 5, 3, 3, 5 dose group configuration and 2 males from pens 2 and 3 were removed in week 6. Pretreatment procedures were performed in week 8, two weeks later than 'Controls'. Behavioural data from macaques in weeks 1 - 5 are compared directly between groups. Baseline physiological data recorded during pretreatment procedures are reported for each Cohort but not compared directly, as groups were no longer matched. Instead, a multiple linear regression model was used to determine the effects of socialisation along with a number of other 'predictor' variables on heart rates and blood pressures from macaques restrained in tubes from seven studies (Table 5.2.7).

Table 5.2.1 Animal demographics.

Cohort	N (M:F)	n/pen	Age (w) SD		BW (Kg)		Acclim (w)	Tube	Manipulation
			M	F	M	F			
'Control'	40 (20:20)	5	118	120	3.1	2.8	6	3	'Control' - experienced normal unit procedures.
'Enhanced'	40* (20:20)	5*	117	120	3.0	2.8	9	3	'Enhanced' - experienced additional socialisation with care staff during daily husbandry and focal socialisation with 1 human carer.

N: Total number of animals included in the study; **M:** Male; **F:** Female; **n/pen:** Number of macaques per pen; **Age:** Age of macaques (weeks) on arrival; **BW:** Mean body weight on arrival (Kg); **Acclim:** Length of acclimatisation time (weeks): time from arrival to day 1 of dose; **Tube:** number of prior habituation sessions in the tube prior to recording; * 'Enhanced' number of males reduced to 16 on study arranged into groups of 5, 3, 3, 5 macaques.

Housing

Animals were housed as described in Chapter 3, Section 3.2.1 and Figures 3.2.1 a - c. 'Control' and 'Enhanced' cohorts were housed in separate rooms.

Husbandry

Husbandry schedules were the same for all macaques, apart from staggered procedures for weighing and physical examination, and consequently being transferred to the playroom. 'Control' macaques were weighed on a Thursday and 'Enhanced' weighed on a Tuesday each week (Table 3.2.2; Section 3.2.1 i & 3.2.1iiif). A full description of animal husbandry procedures are given in Chapter 3, Section 3.2.1. Daily husbandry routines are given in Table 5.2.2; all include visual and auditory contact with care staff and additional hand-feeding of diet (e.g. bonio, supplementary diet). Enhanced socialisation was based around these events.

Table 5.2.2 Daily husbandry schedule for macaques during acclimatisation.

Time/h	Event	Description
08:00	First checks	Visual checks of animals, record room temperature and humidity.
09:30	Husbandry	Visual checks of animals replenish water bottles; feed bonio biscuits, clean aisles.
11:00	Diet	Visual checks of animals scatter feed primate chow.
12:00	Middays	Visual checks of animals.
14.00	Husbandry	Visual checks of animals, replenish water bottles, record temperature and humidity.
15.00	Supplement	Visual checks of animals, feed supplement diet (e.g. fruit, vegetables, forage mix).
16.00	Last checks	Visual checks of animals and pens by two technicians.

5.2.2 Socialisation

The learning processes behind socialisation are given in Table 5.1.1. Food was paired with husbandry events and close contact with care staff (e.g. Clay *et al* 2009) and additional focal sessions were conducted 2 hours three times/week (includes 5 minutes per pen to attach and remove transport box at beginning and end of socialisation session) in the afternoon with the same person (PhD student). The outline plan for socialisation was based upon trials conducted by a small number of care staff who reported its effectiveness in building positive relationships with macaques, and facilitated capture in a transport box and handling during procedures. The PhD work formalised the conduct of socialisation already initiated by care staff by combining positive reinforcement techniques with transport box training and a more structured approach to interaction (Table 5.2.3). Socialisation began three days after arrival, took place during weekdays, continued throughout acclimatisation, and was transferred to technical staff, who resumed normal unit socialisation during dosing for commercial study.

Food was offered from outside of the home pen, through the wide bars in the front of the cage. The schedule for socialisation is outlined below and based around days when forage mix was fed (3 times per week) because it contained a mix of dried fruit and seeds that could be easily handled during hand-feeding. Furthermore, food items perceived to be of higher value (e.g. dried mango, pineapple, apricot) could be used to reward voluntary approach and entry to the transport box. Forage mix was weighed out for each pen, for both Cohorts, each week to ensure macaques were receiving the same allowance and not under- or over- fed. The weighed amounts were checked with senior technicians on the unit before being fed to macaques. A small portion, approximately 10% of forage mix, was placed fresh in a sealed container outside the macaques' home room for technicians to offer during husbandry procedures on three days a week as indicated in Table 5.2.3. Any remaining forage mix at the end of the socialisation session was scattered in the pen as normal. Combinations of different personal protective clothing were worn during socialisation (e.g. overalls, boots, catching gloves, apron and visor).

Table 5.2.3 Socialisation schedule, recording events and description.

Week no.	Day	D	Description	Care staff
1	Fri	2 h	Transport boxes containing forage mix were attached to the front of pens. Focal socialiser remained in the room, stood outside each pen over the central drain at a 45°angle to the pen, but did not make auditory or physical contact for the duration of the session before taking the transport box down. **'Enhanced' and 'Control' human response test 1, staggered at 10 minute intervals between Cohorts (Table 5.2.4). Preformed before socialisation.**	'Enhanced' group: Encouraged to hand feed during morning and afternoon 'husbandry' and 'middays' three days a week. Event recorded in husbandry log.
2	Mon Wed Fri		As above, but transport box contain half forage mix. Focal socialiser offered the remaining half of forage mix to each pen in turn. Forage mix was offered from an open hand through the bars of the pen to macaques on the side bench and veranda (Figure 3.2. 1a – c). The socialiser averted her gaze and spoke softly to the animals. Macaques were specifically rewarded with verbal praise (e.g. "*good-girl; good-boy*") paired with high value food reward for voluntarily entering the transport box, remaining in the transport box and approaching the socialiser. During the last session of the week, small amounts of high value dried fruit in the forage mix were offered from an open gloved hand (e.g. leather catching glove). **Macaques TB tested on respective weigh-days; Tues & Thurs.**	
3	Mon Wed Fri		As above, but transport box contained a quarter of forage mix. Focal socialiser offered the remaining three quarters to each pen in turn as above. In addition and where possible the socialiser gently touched macaques' hands and arms and rewarded macaques for tolerating body touching with higher value dried fruit. In the second session of the week, small amounts of high value dried fruit in the forage mix were offered from an open gloved hand (e.g. leather catching glove) whilst the socialiser wore a visor. **'Enhanced' and 'Control' human response test 2 (Fri), staggered at 10 minute intervals between Cohorts (Table 5.2.4). Performed before socialisation.**	
4	Mon Wed Fri		As above.	
5	Wed Thur Fri		As above, but all forage mix was given from the socialisers hand during the session. Macaques were rewarded for staying in the transport box for longer, staying at the front of the pen in close proximity to the socialiser and tolerating longer and firmer body touching. **Response to handling and pre – and post – handling responses were recorded ('Enhanced': Monday & Tuesday, 'Control': Wednesday & Thursday).** **'Enhanced' and 'Control' human response test 3 (Fri), staggered at 10 minute intervals between Cohorts (Table 5.2.4).**	
6	Mon Wed Fri		As above. But food rewards predominantly given for approaching the socialiser, offered from the finger tips rather than an open hand. **Pretreatment procedures performed in 'Controls'.** **In 'Enhanced' group: 2 males from pens 2 and 3 were removed to give 5, 3, 3, 5 group size for newly assigned study.**	
7	Mon Wed Fri		As above.	
8	Mon Fri		As above. **In 'Enhanced' pretreatment procedures performed on males only.**	
9	-		**On set of dosing. Normal unit procedures for socialisation with care staff continue on commercial study.**	

D: Duration (hours).

'Control' macaques experienced normal unit socialisation procedures. For example, they were desensitised to transport boxes attached to the front of the pen containing forage mix, two hours/three times per week, care staff did not remain in the room beyond placing or taking down the transport box. They were hand-fed bonio biscuit each day and fruit or vegetables twice per week. Care staff also had positive body contact on an *ad hoc* basis during husbandry events (Table 5.2.2).

5.2.3 Human response test

Macaque responses to standardised tests were recorded on weeks 1, 3 and 5 (Table 5.2.3). The procedures for the human response tests include presentation of 'Passive', 'Positive' and 'Capture' tests in succession (Table 5.2.4), partly mimicking the sequence of husbandry events (Table 5.2.2). Simplified behavioural categories were recorded in person, at pen side, on to a check sheet mounted on a clip board (Table 5.2.5). Tests were conducted in the afternoon 30 minutes after 'middays' (Table 5.2.2) and at 30-minute intervals finishing 30 minutes before afternoon 'husbandry'. Overalls and rubber gloves were worn for 'Passive' and 'Positive' test, whilst capture gear was worn (e.g. overalls, apron, visor and 1 leather glove) for 'Capture' tests.

Table 5.2.4 Human response tests recorded in weeks 1, 3 and 5.

Human response test	Procedure
'Passive'	12.30 enter the room. Stand for 60 seconds at the far wall of the room facing the door, not making eye contact or talking to the macaques. Tests are conducted in order of pen number mimicking order of daily checks by care staff. Start test at pen 1. Observer stands over the central drain directly outside the pen, face-on with clipboard and stop watch elevated to comfortable writing position. 30 seconds elapses during which latency to approach the front of the pen at height of side bench and below the veranda is recorded for each macaque. At the end of 30 seconds behaviour is recorded using instantaneous scan sampling method and at 30 second intervals for a further 60 seconds (e.g. 3 time points: T0, T30 and T60). Repeated for pens 2 – 8, end of test. Leave the room for 30 minutes.
'Positive'	Enter the room and as above wait 60 seconds before starting the next test. 25g (a handful) of forage mix was offered from outstretched arm, open palm, palm extending into the pen at the height of the side bench. If one or more macaque(s) sat in front of the hand monopolising the food they were distracted by throwing a large, piece of dried fruit (e.g. mango or pineapple) onto the floor, and remaining food continued to be offered at the original height. Food was offered for 30 seconds before my hand was removed from the pen and 60 seconds of behavioural data was collected as outlined above. Repeated for pens 2 – 8. Leave the room for 30 minutes.
'Capture'	Enter the room wearing full catching gear and as above wait 60 seconds. 25g of forage mix was offered from the gloved hand as described above and the test was conducted following the same procedure for 'Positive'.

Table 5.2.5 Behavioural categories recorded during scan sampling as human response tests.

Categories	Elements	Description
Instantaneous scan sampling every 30 seconds		
Inactive	Embrace	Animal is stationary and in an embrace with another individual. Two individuals clasp each other face-to-face contact between ventral surfaces (chest or head) arms of one or both individuals involved are wrapped around the body of the embrace partner. This may be unilateral or mutual.
	Huddle	Animal is stationary and huddled to another individual: 1 or more surface is in physical contact with another individual.
	Contact	Animal is stationary and awake, and in contact with another individual – within 1 arms reach of another animal.
	Inactive	Animal is stationary; lying, sitting and awake. Animal is not in contact (within 1 arms' reach) with another individual.
Active	Active	Animal moves between locations by walking, climbing, running or swinging.
Location	Veranda	Animal on the veranda
	Upper	Animal is in the upper half of the pen.
	Lower	Animal is in the lower half of the pen.
	Floor	Animal on the floor of the pen.
	Front	Animal is in the front half of the pen.
	Back	Animal is in the back half of the pen.
All occurrences		
Vocalisation	Kra, Kra Alarm, Wraagh, Scream, Khreet Screech, Squeal, Whimper, Coo	Described in Table 3.2.8.
Aggression	Directed towards cage mates	Animal engages cages in bodily contact or facial threat directed towards another individual. May include chase, slaps, hair pulling, open-mouthed display, direct stare.
	Direct towards cage	Any vigorous shaking or banging of the cage with feet (most common) or hands.
Others	Other	Any behaviour not listed in the above descriptions. Describe form and context.
	Comments	Comments regarding events in the environment or problems with recording.

5.2.4 Welfare assessment and toxicological methods and recording

Table 5.2.6 gives the framework, methods and schedule of recording for welfare assessment and toxicological recording. Note the methods are fully described elsewhere (e.g. Chapter 3).

Table 5.2.6 Welfare assessment framework and toxicological recording and methods.

Variable	Recording & analysis method	Detailed method given
Welfare variable		
Body condition score (BCS)	Recorded weekly by PhD student, not on arrival.	Clingerman & Summers (2005). Table 3.2.2. Section 3.2.2 (i) a. Table 3.2.3.
Alopecia score (APS)	Recorded weekly by PhD student, not on arrival. Intra-observer reliability recorded for body condition and alopecia scores in week 2 with TB testing.	Honess *et al* (2005). Table 3.2.2. Section 3.2.2 (i) b. Table 3.2.4.
Body weight (BW)	Recorded weekly by care staff including arrival.	Table 3.2.2. Section 3.2.2 (i) d.
Clinical observation (CO)	Animals physically examined on arrival and weekly by care staff.	Table 3.2.2. Section 3.2.2 (i) c.
Behavioural observations (BO) a) During handling and restraint for weighing and physical examination	Week 5 ('Controls' & 'Enhanced').	Table 3.2.2. Section 3.2.2 (ii) f. Table 3.2.5. Table 3.2.8.
b) Pre – and post – handling in the home pen	Week 5 ('Controls' & 'Enhanced').	Table 3.2.2. Section 3.2.2 (ii) h. Table 3.2.9a – c.
c) During recording ECG and HDO	Week 6 ('Controls'). Week 8 ('Enhanced').	Section 3.2.2 (ii) g. Table 3.2.6. Table 3.2.8.
Human response test	Weeks 1, 3 & 5 ('Controls' & 'Enhanced').	Section 5.2.3. Table 5.2.4. Table 5.2.5.
Toxicological measures		
Electrocardiogram (ECG) measurement of heart rate (HR/ bpm)	Week 6 ('Controls'). Week 8 ('Enhanced'). Animals were habituated to tubes for 1 minute each day for 3 consecutive days before recording.	Table 3.2.2. Section 3.2.2 (ii) e. Appendix 1.3.
High definition oscillometry (HDO) measurement of blood pressure (BP: SYS; DIA; MAP/ mm/Hg)	Week 6 ('Controls'). Week 8 ('Enhanced'). Animals were habituated to tubes for 1 minute each day for 3 consecutive days before recording. Time taken and number of attempts to record 5 blood pressure measurements were recorded.	Table 3.2.2. Section 3.2.2 (ii) e. Appendix 1.5.

5.2.5 Data analysis

All data were examined for normality and transformed (Section 4.2.6 a & c) if possible to permit parametric statistical analysis; if normality could not be achieved with transformation non parametric statics were used where indicated. Independent *t-tests* were used to determine differences in age and body weight on arrival between male and female macaques and between Cohorts. To identify changes in body weight with acclimatisation and pretreatment procedures, repeated measures Analysis of Variance (ANOVA) with Bonferroni *post hoc* tests and sex as a between-subject factor were performed on each Cohort. Univariate analyses, run with sex as a covariate, were performed to compare weekly changes in body weight between Cohorts. To determine the intra-observer reliability for pairs of body condition and alopecia scores recorded

during TB testing, the percentage of agreement was calculated. Repeated measures Analysis of Variance (ANOVA) with sex as a between-subject factor and Bonferroni *post hoc* tests were performed to compare behaviours in the pre – and post – handling observations in week 5. Human response test data could not be transformed to improve normality, therefore Wilcoxon signed-rank test was used to compare responses in week 3 and week 5, and Mann-Whitney U- test was used to compare 'Control' and 'Enhanced' macaques for test response.

Male-only data were included for analysis of heart rates and blood pressures. The confounding effects of extra time spent in the unit (e.g. week 8 vs. week 6 for ECG and HDO recording in 'Enhanced' vs. 'Controls' respectively) meant a between-subjects statistical approach was not suitable for analysis. As an alternative a multiple linear regression model (Section 4.2.9; Table 5.2.7) was used to determine the effects of multiple predictors (e.g. socialisation, acclimatisation, tube habituation, age etc.) on variation in heart rates from the first seven studies where tube restraint was used by the CASE sponsor, and includes data collected from Studies 1, 2 and 3 in Chapter 3. Blood pressure measurements were recorded in five out of the seven studies (including Studies 1 and 2 in Chapter 3,) and these were entered in to a similar multiple regression model (Table 5.2.7).

Table 5.2.7 Multiple linear regression model for determining the variability of cardiovascular parameters.

N	Outcome variable				Predictor variable			
	Variable	**Unit**	**Description**	**Regression model**	**Variable**	**Unit**	**Description**	**Regression model**
132	HR	bpm	Continuous	Entered as continuous data	Age	W	Continuous	Entered as continuous data
					Acclimatisation	W		
					Tube habit	-	Categorical	Count
					Enhanced	-	Discrete	Yes (0) No (1)
84	BP	mm/Hg			Age	W	Continuous	Entered as continuous data
					Acclimatisation	W		
					Tube habit	-	Categorical	Count
					Enhanced	-	Discrete	Yes (0) No (1)
					Time	min	Continuous	Entered as continuous data
					No. of recordings	-	Categorical	Count

N: Number of macaques; **HR:** Heart rate (bpm); **BP**: Blood pressures (systolic, diastolic, mean arterial); **Acclimatisation:** from arrival to day 1 of dose; **Tube habit:** Number of tube habituations prior to baseline recording; **Enhanced:** Macaques experienced enhanced socialisation; **Age:** Age in weeks at start of study; **Time:** Time taken to record all blood pressures; **No. of recordings:** Number of attempts to take 5 blood pressure recordings.

5.3 Results

5.3.1 Welfare

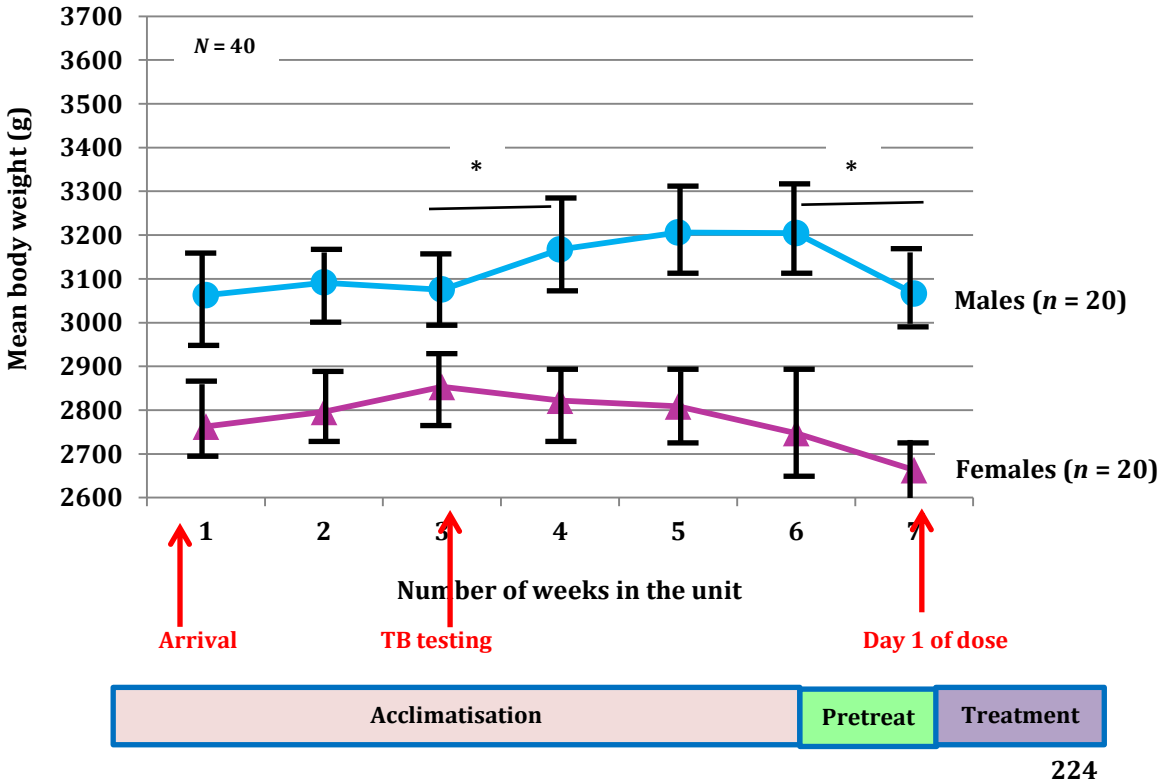
There were no significant differences in age of macaques on arrival. Animals were in good health apart from those with hair loss and being underweight recorded by care staff and in agreement with my observations. Three females in the 'Enhanced' group had diarrhoea in week 1, accounting for the initial weight loss observed in these animals. They did not require treatment and diarrhoea quickly resolved.

5.3.2 Change in body weight, condition and alopecia

a) Body weight

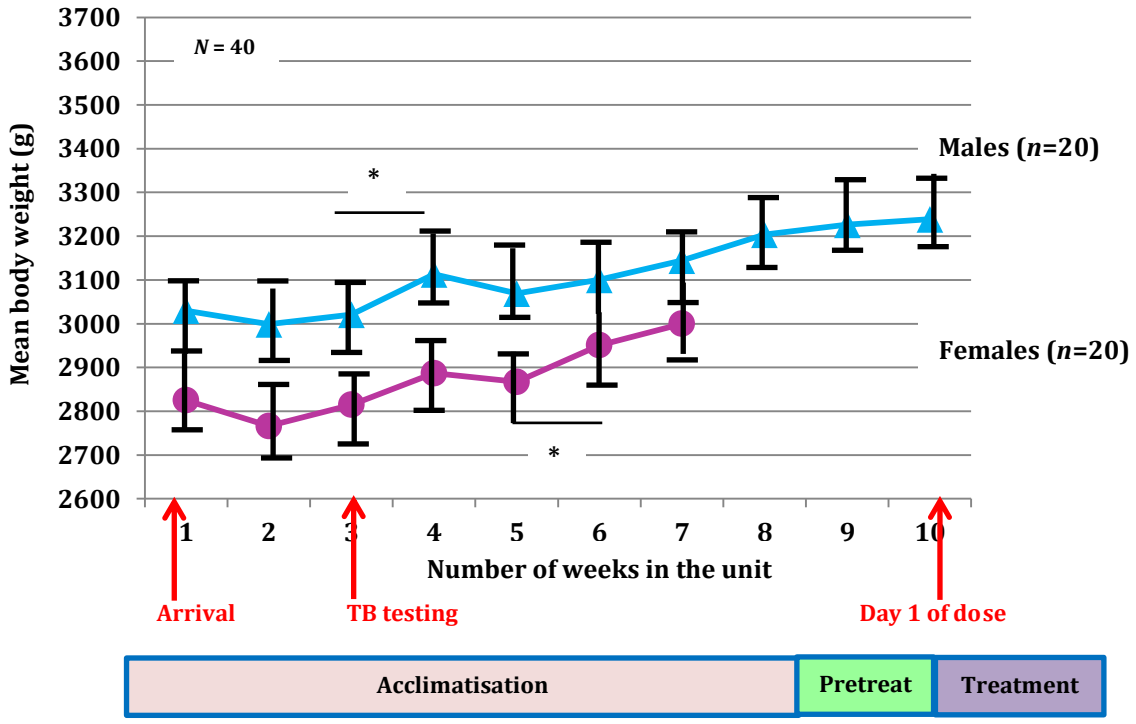
Male and female macaques showed similar body weights on arrival in 'Control' and 'Enhanced' groups. Males were significantly heavier than females in both Cohorts ('Controls' males: mean (g): 3062 ± 184.9, females: mean(g): 2762 ±223.5, $t(39) = 4.30$, $p<0.05$; 'Enhanced' males: mean (g): 3030 ± 295.8, females: mean(g): 2825 ± 168.2, $t(39) = 4.53$, $p<0.05$). 'Control' animals did not significantly gain weight overall from arrival to day 1 of dose. Indeed females weighed less on day 1 of dose than they did on arrival ($f(1) = 3.86$, $p<0.05$), whereas both males and females were heavier by week 6 of acclimatisation in the 'Enhanced' group (males: $f(1) = 12.37$, $p<0.05$; $f(1) = 14.57$, $p<0.05$). Furthermore, males continued to grow even during pretreatment procedures, and were significantly heavier by week 9 after arrival ($f(1) = 3.86$, $p<0.05$).

Figures 5.3.2 a Fluctuations in body weights of males and females during acclimatisation and pretreatment period in 'Control' (i) and 'Enhanced' (ii) groups.
(i) 'Control'



In the 'Control' group females did not show significant weight changes week-to-week. Males gained weight significantly in weeks 2 – 3 (mean: + 3.0%; $f(1) = 28.63$, $p<0.05$) and significantly lost weight in weeks 5 – 6 at the onset of pretreatment procedures (mean: - 4.3 %; $f(1) = 29.72$, $p<0.05$). In week 5 'Control' males were heavier than 'Enhanced' males ($t(39) = 4.06$, $p<0.05$), although this relationship was reversed in week 7, due to pretreatment weight loss observed in 'Control' males ($t(39) = 4.92$, $p< 0.05$). Females of 'Control' and 'Enhanced' groups showed a different pattern of change, with 'Enhanced' females heavier than control group from week 4 onwards (week 4: $t(39) = 9.16$, $p<0.05$).

(ii) 'Enhanced'



'Enhanced' females were not followed on to study, so data from week 6 of acclimatisation are not reported. Males and females showed a similar pattern of weight change week-to-week. Males and females gained significant weight in weeks 3 - 4 (males: $f(1) = 11.43$, $p<0.05$; females: $f(1) = 16.03$, $p<0.05$), and females also displayed significant weight gain in weeks 5 – 6 ($f(1) = 16.03$, $p<0.05$).

b) Intra-observer reliability of body condition and alopecia scores

The intra-observer reliability of body condition and alopecia scores from repeated scoring of the same animals during TB testing are given in Table 5.3.1.

Table 5.3.1 Intra-observer reliability of body condition and alopecia scoring methods.

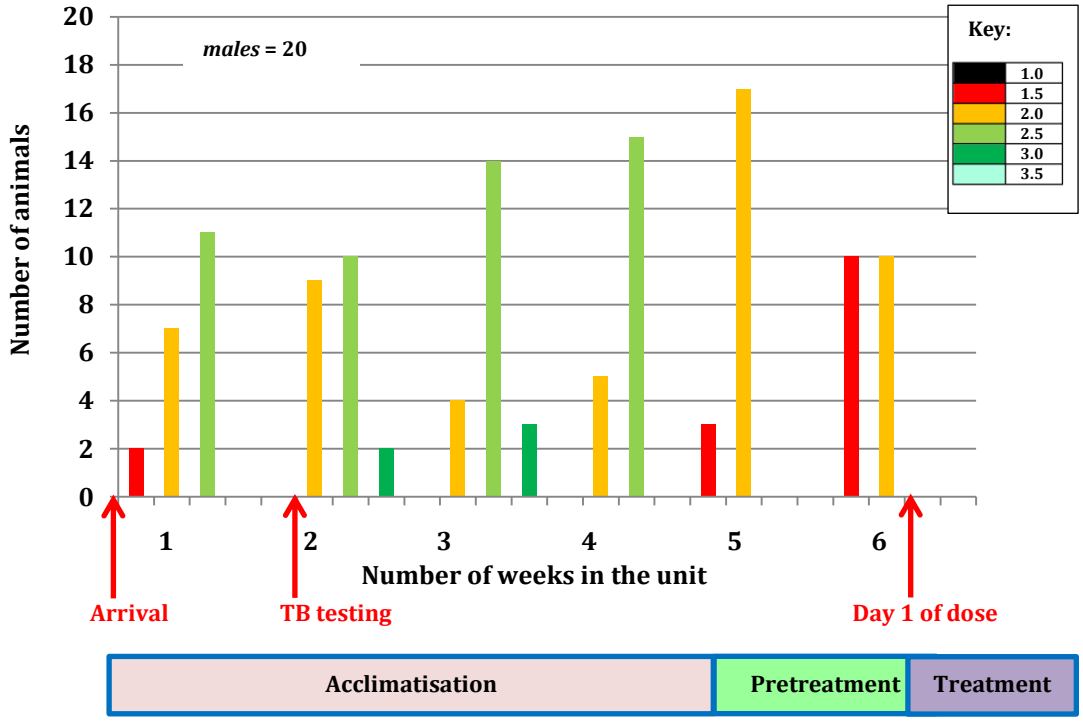
Cohort	N	Score method	No of scores in agreement/disagreement (distance)	% agreement
'Control'	40	Body condition (Clingerman & Summers 2005)	38/40 (± 0.5)	95
	40	Alopecia (Honess *et al* 2005)	40/40 (0)	100
'Enhanced'	40	Body condition (Clingerman & Summers 2005)	39/40 (± 0.5)	98
	40	Alopecia (Honess *et al* 2005)	40/40 (0)	100

Distance: the magnitude of the disagreement between scores. **N:** number of macaques scored.

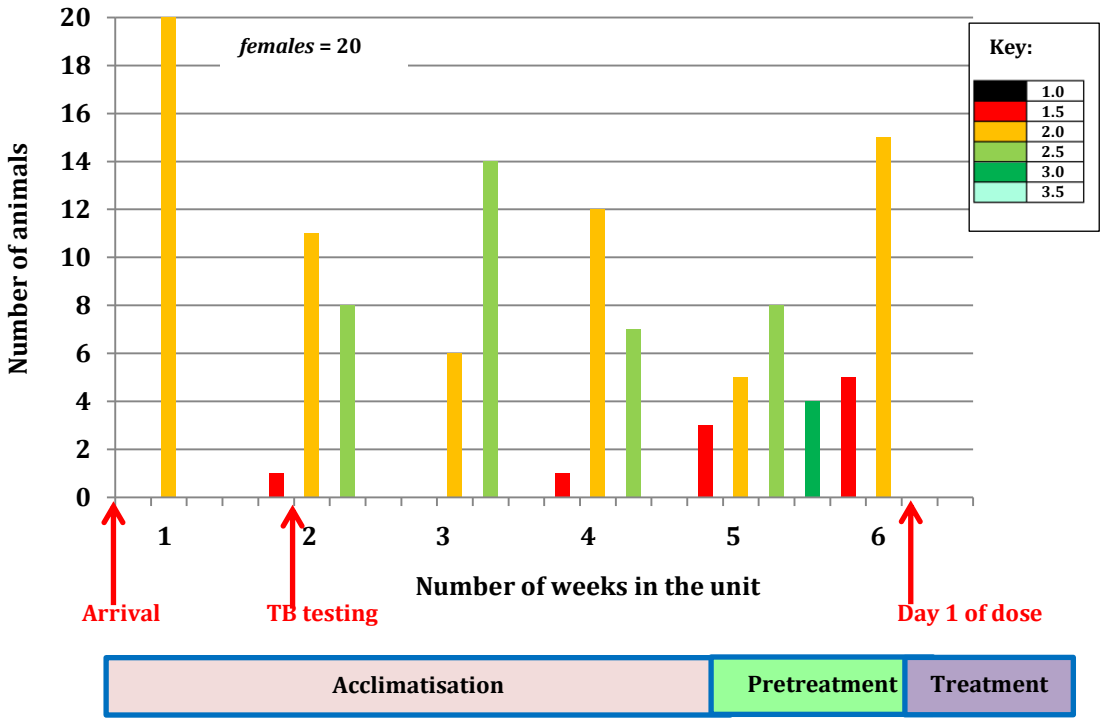
Percentage agreement for all scores were high in both Cohorts.

c) Body condition (Clingerman & Summers 2005)

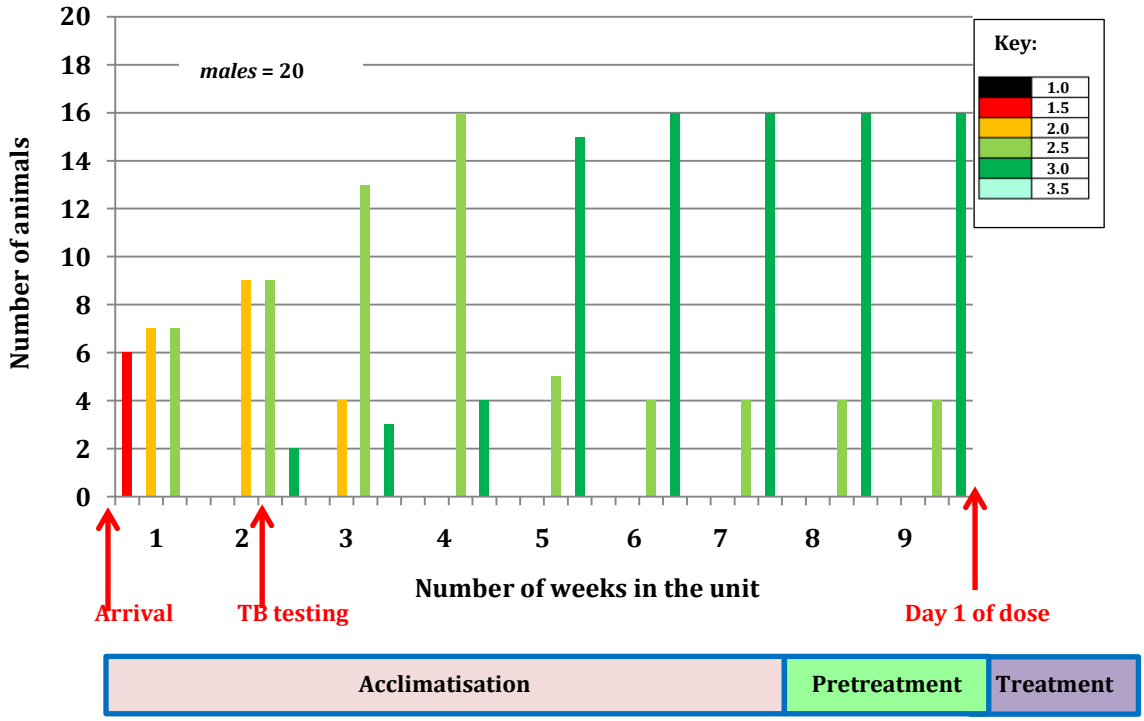
Figures 5.3.2 b Change in body condition of males and females during acclimatisation and pretreatment in 'Control' (i) and 'Enhanced' (ii) groups.
(i) 'Control'



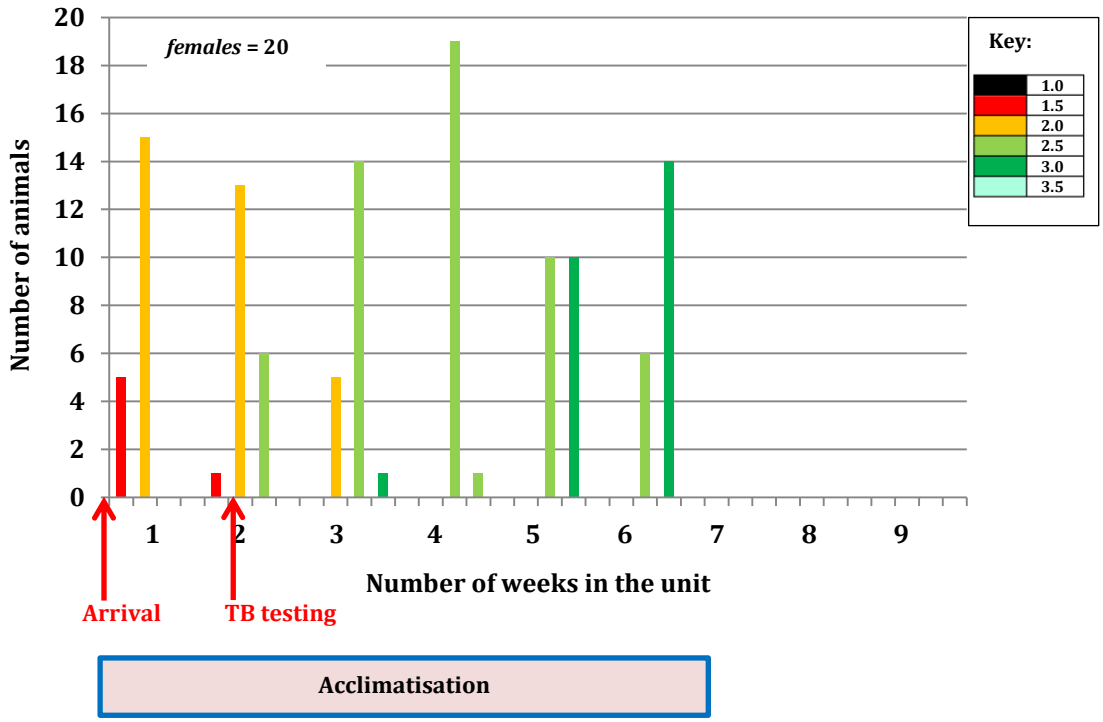
Macaques were not body condition scored on arrival. Body condition of males in the 'Control' group increased with time in the unit until week 5 and the onset of pretreatment procedures following weight loss. Females were slower than males to gain body condition following a similar trend with body weight gain. More females were very thin (e.g. 1.5; *n* = 5) at start of dosing than during week 1 on the unit.



(ii) 'Enhanced'



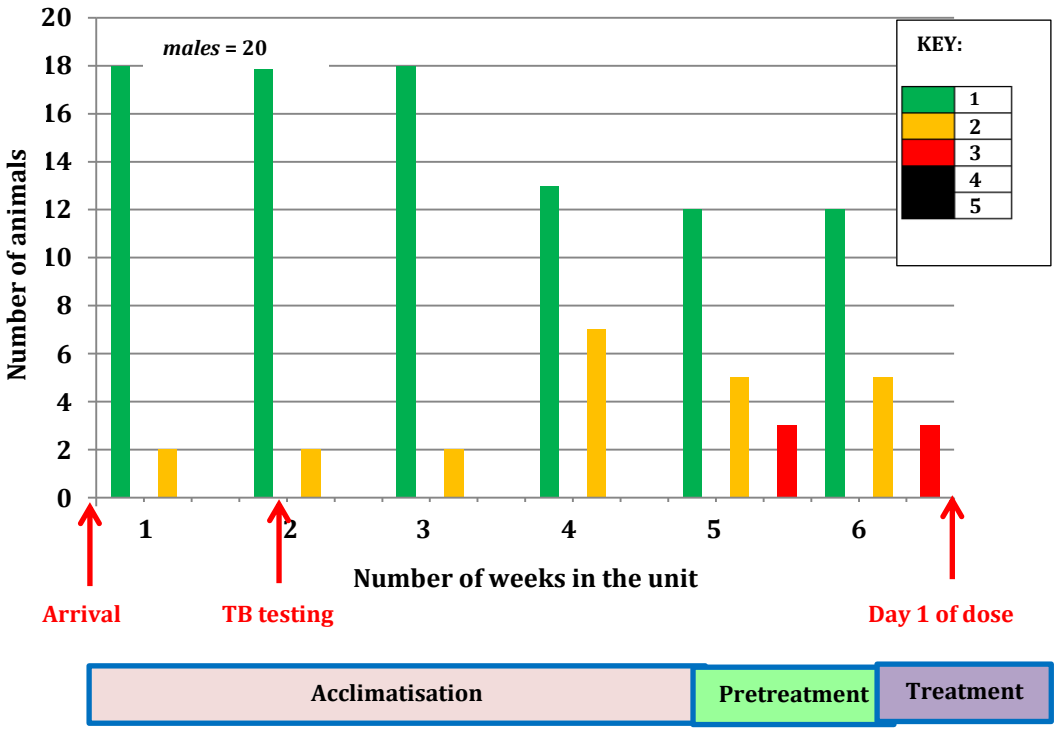
Body condition of both males and females improved with time spent in the unit. The majority of males were at optimum body condition (e.g. score 3) at start of study in week 9. Furthermore, more animals had better body condition (males and females) in the 'Enhanced' group compared to 'Control' group in weeks 4, 5 and 6.



'Enhanced' females were not followed on to study and data from week 6 of acclimatisation are not reported.

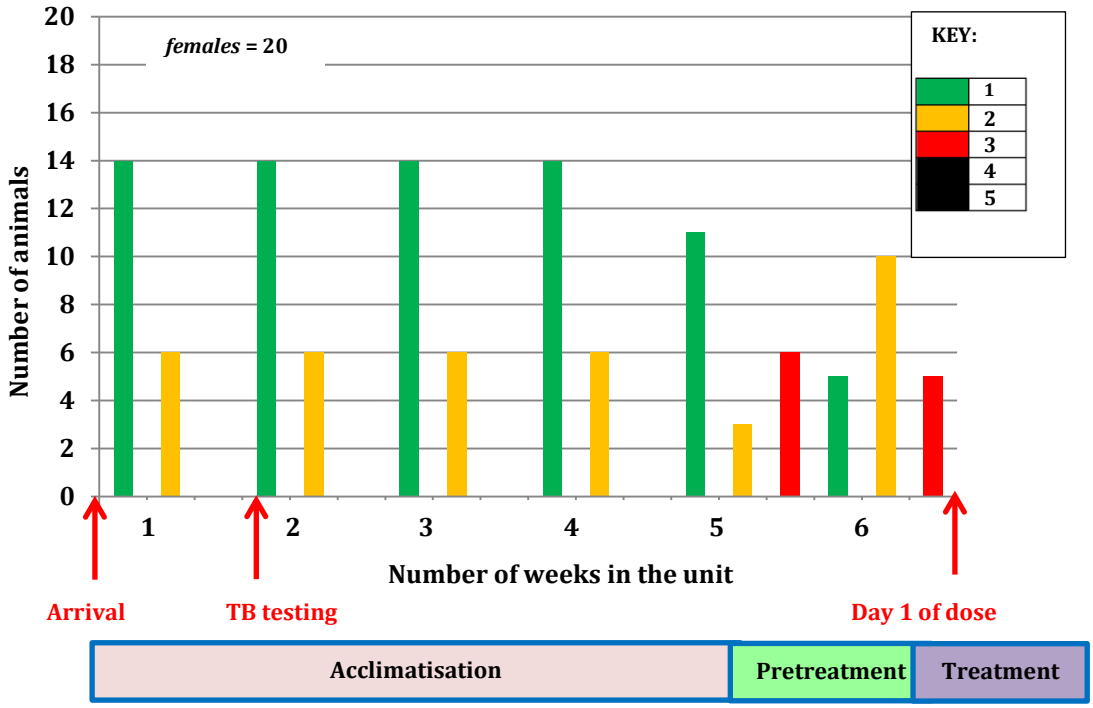
d) Alopecia (Honess *et al* 2005)

Figure 5.3.2 c Change in alopecia scores of males and females during acclimatisation and pretreatment in 'Control' (i) and 'Enhanced' (ii) groups.
(i) 'Control'



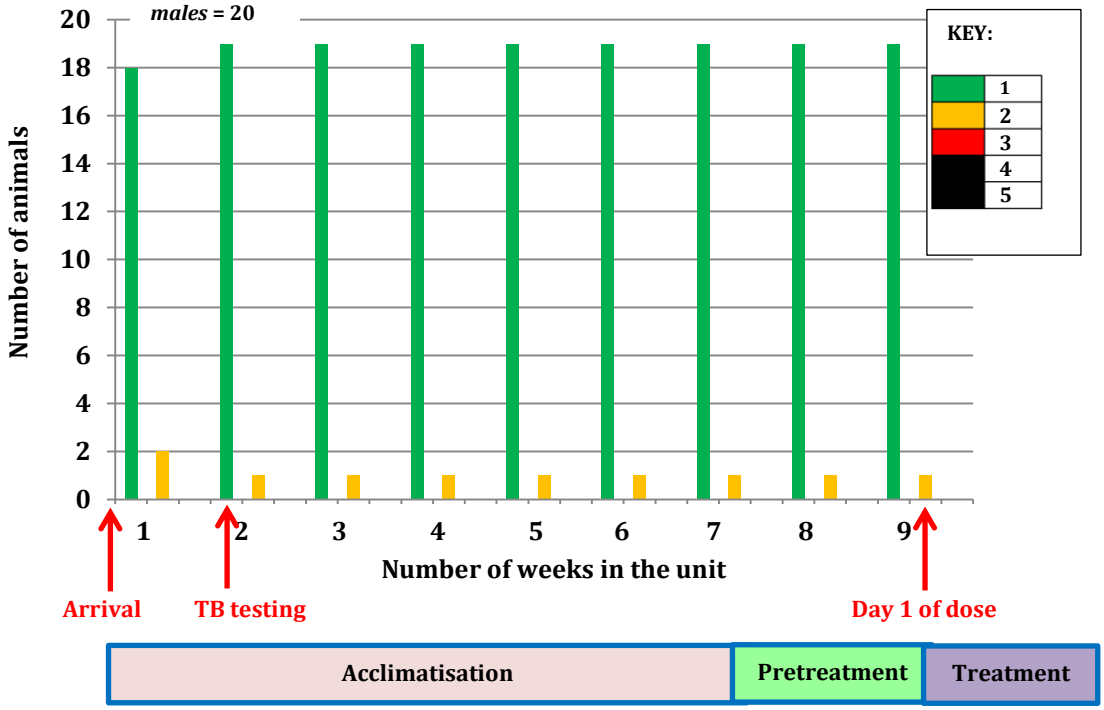
Alopecia was not scored on arrival. There were differences over time in the pattern of hair loss in both male and female 'Control' animals. Between weeks 5 - 6 the number of animals with large

patches of hair loss (e.g. grade 3) increased from 0 to 3 in males (15 %) and 0 to 6 in females (30 %).

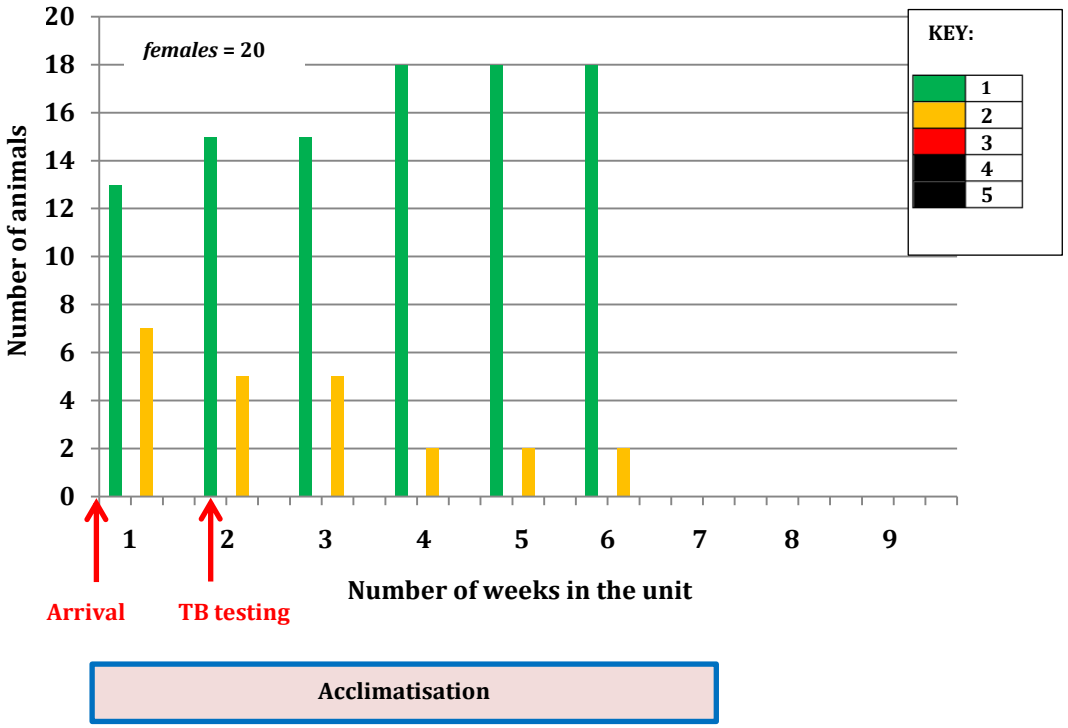


The pattern of hair loss between 'Control' and 'Enhanced' animals was different. The incidence and severity of hair loss were found to be greater in 'Control' animals from week 5 onwards, coinciding with pretreatment procedures in these animals. Note that the majority of 'Enhanced' animals maintained normal pelage throughout their pretreatment period, week 7 onwards.

(ii) 'Enhanced'



There were no differences in incidence and severity of hair loss over acclimatisation time in males, whilst females showed improvement in pelage condition with time spent in the unit.

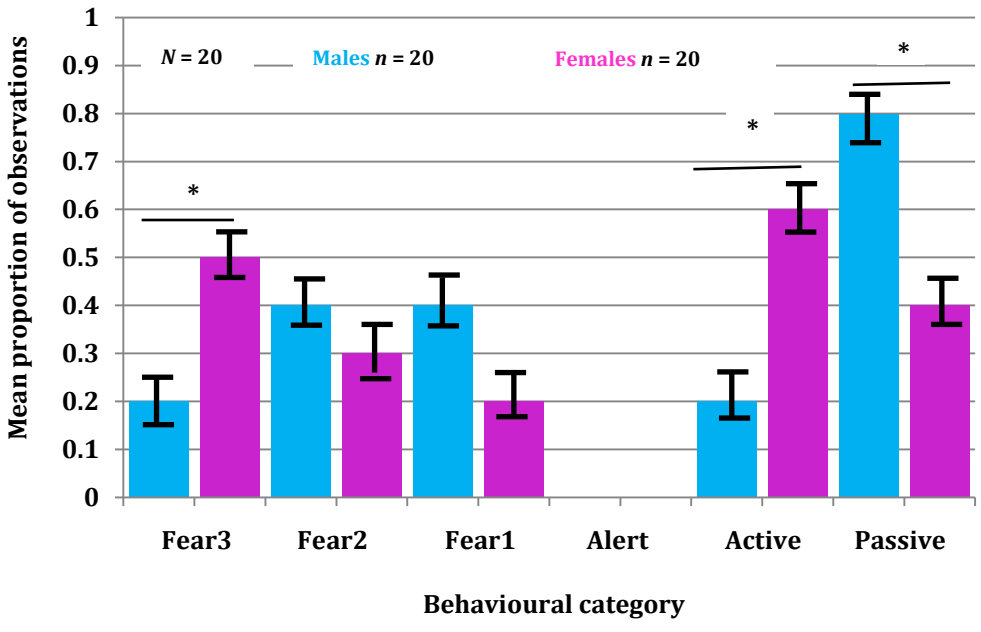


'Enhanced' females were not followed on to study and data from week 6 of acclimatisation are not reported.

5.3.3 Behavioural responses

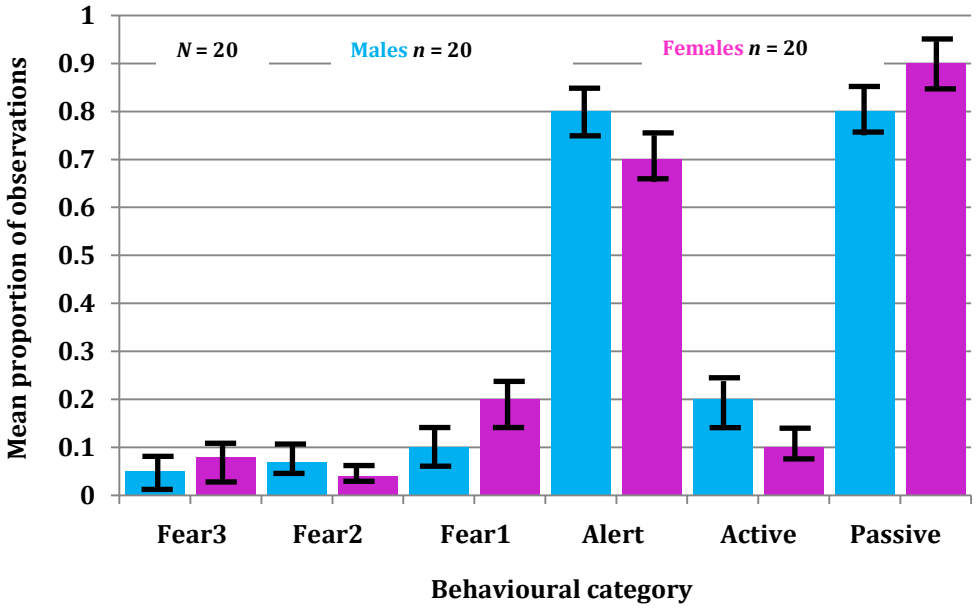
a) During handling and restraint in week 5

Figure 5.3.3 a Behavioural responses of males and females during handling and restraint for weighing and physical examination in week 5 in 'Control' (i) and 'Enhanced' (ii) groups.
(i) 'Control'



The behavioural responses of male and female macaques in 'Control' group during handling for weighing and physical examination in week 5 differed significantly. Females showed a greater proportion of Fear3 facial expression than males ($t(39) = 4.43$, $p<0.05$) and greater activity ($t(39) = 3.50$, $p<0.05$). By contrast, males were more passive during handling ($t(39) = 3.51$, $p<0.05$). The responses of 'Control' and 'Enhanced' macaques differed significantly on all behavioural categories. 'Control' animals were more fearful (Fear3 $f(1) = 5.21$, $p<0.05$; Fear2 $f(1) = 5.99$, $p<0.5$; Fear1 $f(1)$ 2.13, $p<0.05$) and more Active (Active $f(1) = 4.10$, $p<0.05$; Passive $f(1) = 4.10$, $p<0.05$) than 'Enhanced' during handling.

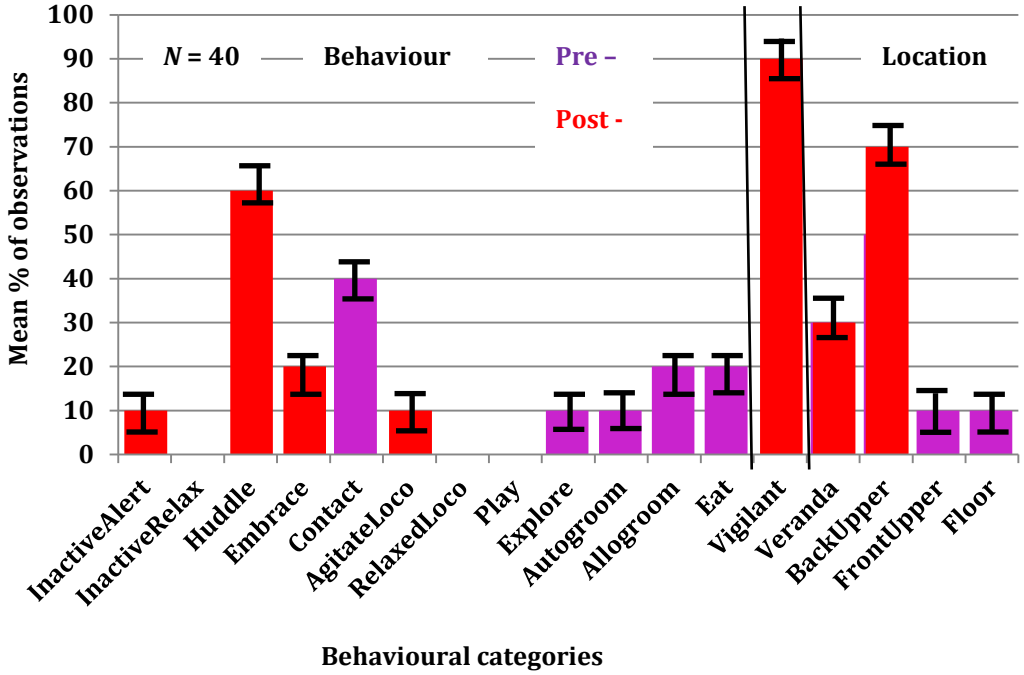
(ii) 'Enhanced'



There were no significant differences between male and females in 'Enhanced' group ($p>0.05$). Animals were predominantly Alert and Passive during handling.

b) Pre – and post – handling for weighing and physical examination in week 5

Figure 5.3.3 b Behaviour of macaques pre – and post – handling for weighing and physical examination in 'Control' (i) and 'Enhanced' (ii) groups.
(i) 'Control'

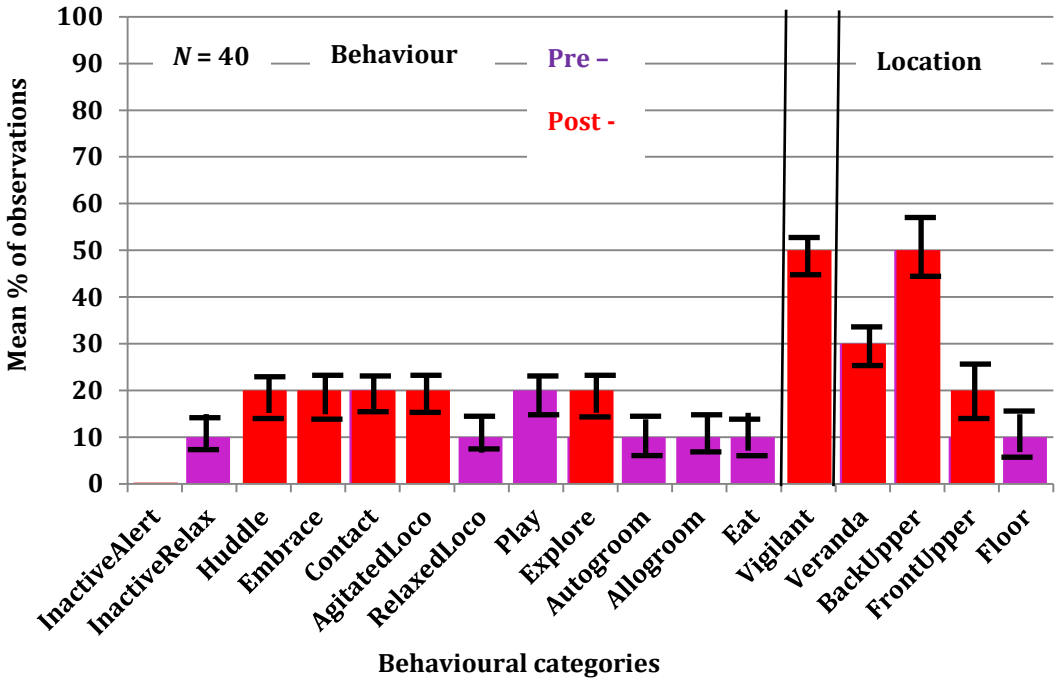


In 'Control' animals there were no differences in males and females in pre – or post - handling observations and no differences between hour 1 and hour 2 in pre - or post – handling recording sessions. Responses of macaques pre – and post – handling differed for every behavioural category (p<0.05). Macaques' behavioural repertoire pre – handling was broader with contact, explore, auto, allo groom and feeding/foraging seen in pre – but not post – handling. Post – handling, macaques were vigilant and spent most of the recording session huddling. They also spent a greater proportion of observations in the back upper portion of the pen.

There were significant differences between 'Control' and 'Enhanced' in pre – and post – handling observations. In pre – handling, Inactive relaxed (f(1) = 2.37, p<0.05), relaxed locomotion (f(1) = 2.24, p<0.05), in contact (f(1) = 5.58, p<0.05), play (f(1) = 5.01, p<0.05) and allo-groom (f(1) = 3.28, p<0.05) significantly differed between Cohorts. 'Enhanced' group spent more time inactive relaxed, relaxed locomotion and play than 'Control', whereas 'Control' animals spent more time in contact and allo – grooming. Differences between Cohorts in post – handling observations included inactive alert (f(1) = 2.67, p<0.05), huddle (f(1) = 5.36, p<0.05), contact (f(1) = 3.51, p<0.05), exploration (f(1) = 2.22, p<0.05) and vigilance (f(1) = 7.60, p<0.05). 'Enhanced' animals had a wider

behavioural repertoire post – handling when compared to 'Control' animals – they spent less time Inactive alert, huddling, being vigilant and more time exploring and in contact; this difference was due to changes in hour 1 and hour 2 (see below).

(ii) 'Enhanced'



There were no differences between male and female macaques in 'Enhanced' group (p>0.05). Macaques had a more varied behavioural repertoire pre – handling compared to post – handling. Post – handling behaviours differed on all behavioural categories (p<0.05) but Contact. In the post – handling observations, macaques spent more time huddling, embracing and in agitated locomotion being vigilant compared to matched time points pre – handling. In the two hours following handling, huddle responses significantly declined (f(1) = 2.45, p<0.05), whilst Explore increased (f(1) = 7.00, p<0.05) between hour 1 and hour 2.

c) Night-time behaviours pre – and post – handling for weighing and physical examination in week 5

Pre – and post – night-time behavioural responses for 'Control' and 'Enhanced' groups are given in Tables 5.3.3 a – c. There were no significant differences between males and females in either Cohort.

Table 5.3.3 a Night-time pre – and post – handling behavioural observations in week 5 for 'Control' and 'Enhanced groups.

Cohort	Observations	Before midnight (18.30 – 23.30h)			Midnight onwards (00.00 – 05.30h)		
		Percentage of scan samples (%)			Percentage of scan samples (%)		
		Asleep	Awake	Active	Asleep	Awake	Active
'Control'	Pre -	93.7	7.3	0.0	76.3	13.3	10.4
'Enhanced'	Pre -	96.0	3.3	0.0	90.3	2.7	7.0
'Control'	Post -	80.0	20.0	0.0	60.1	19.9	20.0
'Enhanced'	Post -	87.5	12.5	0.0	66.5	13.3	20.0

Observation: Pre - /post – behaviour observations in relation to handling & physical examination by technician (Table 3.2.9 a); **Asleep, Awake, Active**: Defined in Table 3.2.9 c. Male & female data pooled.

Table 5.3.3 b Pairwise comparisons within-groups pre - and post - handling night–time behaviours.

Behaviour	'Control'				'Enhanced'			
	Before midnight (18.30 – 23.30h)		Midnight onwards (00.00 – 05.30h)		Before midnight (18.30 – 23.30h)		Midnight onwards (00.00 – 05.30h)	
	Pre -	Post -	Pre -	Post -	Pre -	Post -	Pre -	Post -
Asleep	$f(1) = 31.99$ $p<0.05$		$f(1) = 49.23$ $p<0.05$		$f(1) = 19.29$ $p<0.05$		$f(1) = 8.49$ $p<0.05$	
Awake		$f(1) = 16.89$ $p<0.05$				$f(1) = 36.03$ $p<0.05$		$f(1) = 38.68$ $p<0.05$
Active				$f(1) = 38.85$ $p<0.05$				$f(1) = 11.40$ $p<0.05$

denotes % scan sample observations were significantly higher.

Table 5.3.3 c Pairwise comparisons between-groups pre – and post – handling night-time behaviours.

Behaviour	Pre - handling				Post - handling			
	Before midnight (18.30 – 23.30h)		Midnight onwards (00.00 – 05.30h)		Before midnight (18.30 – 23.30h		Midnight onwards (00.00 – 05.30h)	
	'Control'	'Enhanced'	'Control'	'Enhanced'	'Control'	'Enhanced'	'Control'	'Enhanced'
Asleep				$f(1) = 5.39$ $p<0.05$		$f(1) = 14.79$ $p<0.05$		$f(1) = 13.96$ $p<0.05$
Awake				$f(1) = 6.74$ $p<0.05$	$f(1) = 37.72$ $p<0.05$		$f(1) = 15.79$ $p<0.05$	
Active								

denotes % scan sample observations were significantly higher.

There were no differences between Cohorts before midnight in pre-handling observations during week 5 acclimatisation. Macaques in the 'Enhanced' group spent more time asleep before handling after midnight than 'Controls'. Furthermore, after handling 'Enhanced' macaques spent more time asleep and less time awake before and after midnight than 'Control' animals. 'Controls' were awake more before and after midnight post – handling.

d) Reactivity to humans

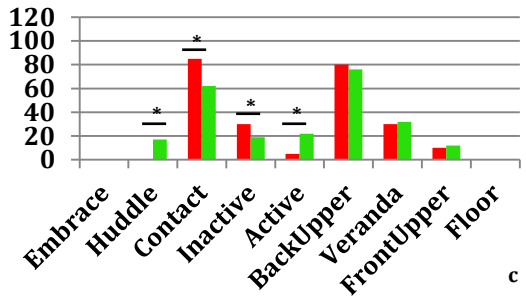
The behavioural responses of macaques in 'Control' and 'Enhanced' groups during weeks 3 and 5 to three successive human response tests: 'Passive', 'Positive' and 'Capture' are shown in Figures 5.3.3 c –h.

Figures 5.3.3 c –h Beahviours recorded during human response tests: 'Passive', 'Positive' and 'Capture'in 'Control' and 'Enhanced' groups.

Passive

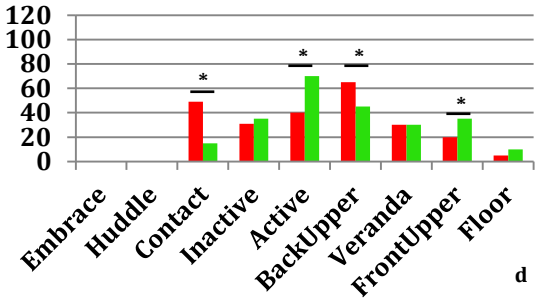
(i) 'Control'

Total observations



(ii) 'Enhanced'

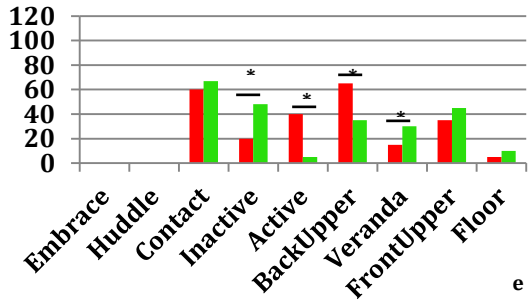
Total observations



Positive

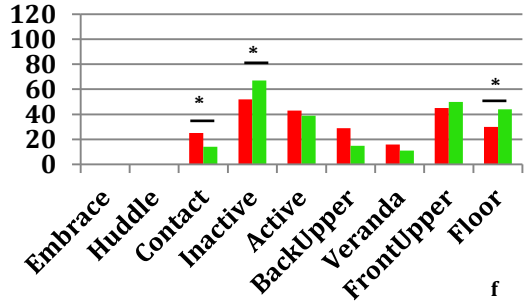
(i) 'Control'

Total observations



(ii) 'Enhanced'

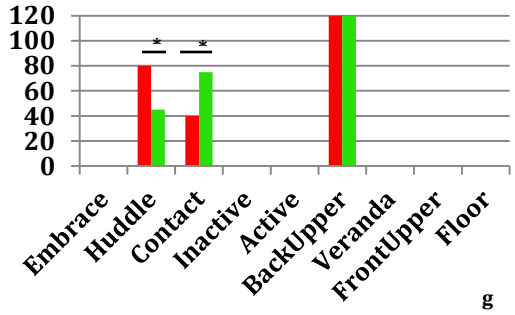
Total observations



Capture

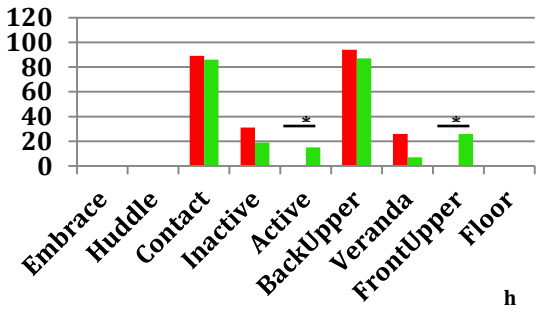
(i) 'Control'

Total observations



(ii) 'Enhanced'

Total observations



Week 3 **Week 5; *N* = 40; males = 20, females = 20. Passive, Positive and Capture are described in Table 5.3.4.**

Week 1 observations were not included as macaques did not approach the human observer or take food in the response tests. Futhermore, they remained huddled at the back upper part of the pen throughout recording sessions. The responses were identical in both Cohorts across tests.

'Control' macaques showed significant diffferences over time in the human response tests. For example, during the passive test macaques in week 5 spent more time huddling and active and less time in contact and inactive (huddle: z = -1.61, p<0.05; contact: z = - 1.76, p<0.05; inactive: z = -1.76, p<0.05; active: z = -1.68, p<0.05). When exposed to the positive human test in week 5 macaques spent more time inactive and on the veranda, and less time active and on the upper back part of the pen compared to their responses in week 3 (inactive: z = -1.62, p<0.05; active: z = - 2.12, p<0.05; back upper: z = -1.93, p<0.05; veranda: z = -1.71, p<0.05). During the capture test there were no differences in macaque's location in the home pen between weeks 3 and 5, but macaques spent more time in contact and less time huddling in week 5 (contact z = -1.67, p<0.05; huddle: z = -1.67, p<0.05).

Macaques in the 'Enhanced' groups also showed differences in their reaction to the three human response tests with time spent in the unit. During the first, passive test, macaques in week 5 spent less time in contact and more time active compared to week 3 (contact: z = -2.41, p<0.05; active: z = -1.83, p<0.05). Whilst in the positive test they spent less time in contact and more time inactive and on the floor of the pen (contact: z = -1.76, p<0.05; inactive: z = -1.85, p<0.05; floor: z = -1.63, p<0.05). During the final capture test they were more active (z = -1.84, p<0.05) and in the front upper (z = -1.66, p<0.05) part of the pen.

There were also significant differences between Cohorts in responses to the three tests in both week 3 and week 5. In week 3 during the passive test 'Enhanced' animals showed less contact (z = -1.74, p<0.05) and more active (z = -1.83, p<0.05) and fewer sample points in the back upper of the pen (z = -1.80, p<0.05). A similar pattern of difference was observed in the postive test, with 'Enhanced' animals showing less contact (z = -1.89, p<0.05) andgreater activity (z = -1.74, p<0.05); they were also seen more often on the floor (z = -2.10, p<0.05) and front upper (z = - 1.98, p<0.05) part of the pen compared to 'Control' animals. During capture 'Control' macaques spent more time

huddling, and less time in contact than 'Enhanced'. Furthermore, 'Enhanced' spent more time on the veranda and less time at the back upper part of the pen compared to 'Control'. The pattern of differences in week 5 observations was different than in week 3. In the passive tests 'Control' animals spent more time huddling (z = -1.74, p<0.05) or in contact (z = - 3.03, p<0.05) than enhanced animals and in the upper back (z = -2.09, p<0.05) portion of the pen. During the positive human response test 'Controls' spent more time in contact (z = -2.27, p<0.05) on the upper back (z = -1.69, p<0.05) of the pen and less time inactive (z = -1.67, p<0.05) and active (z = -2.94, p<0.05) on the veranda (z = -1.88, p<0.05) or floor (z = -2.80, p0.05) of the pen than the 'Enhanced' group. 'Controls' were found to huddle (z =-1.69, p<0.05) more when exposed to capture test than 'Enhanced'. In contrast, 'Enhanced' spent more time inactive (z = -2.09, p<0.05) and active (z = -1.86, p<0.05) on the veranda (z = -1.76, p<0.05) and front upper (z = -3.32, p<0.05) than controls.

Latency to approach

Macaques in both groups and in both testing weeks (i.e. 3 & 5) did not approach during 30 seconds in the passive or capture test. In week 3 during positive test 'Control' animals took longer to approach than 'Enhanced' (mean 'Control' 12.0s; 'Enhanced' 8.3s, t(79) = 2.22, p<0.05). Both groups were faster to approach during positive tests in week 5 compared to week 3 ('Control' t(39) = 2.20, p<0.05; 'Enhanced' t(39) = 2.00, p<0.05) but 'Enhanced' took significantly less time to approach than 'Controls' (mean: 'Control' 8.0s; 'Enhanced' 6.1s, t(79) = 7.61, p<0.05).

Vocalisations

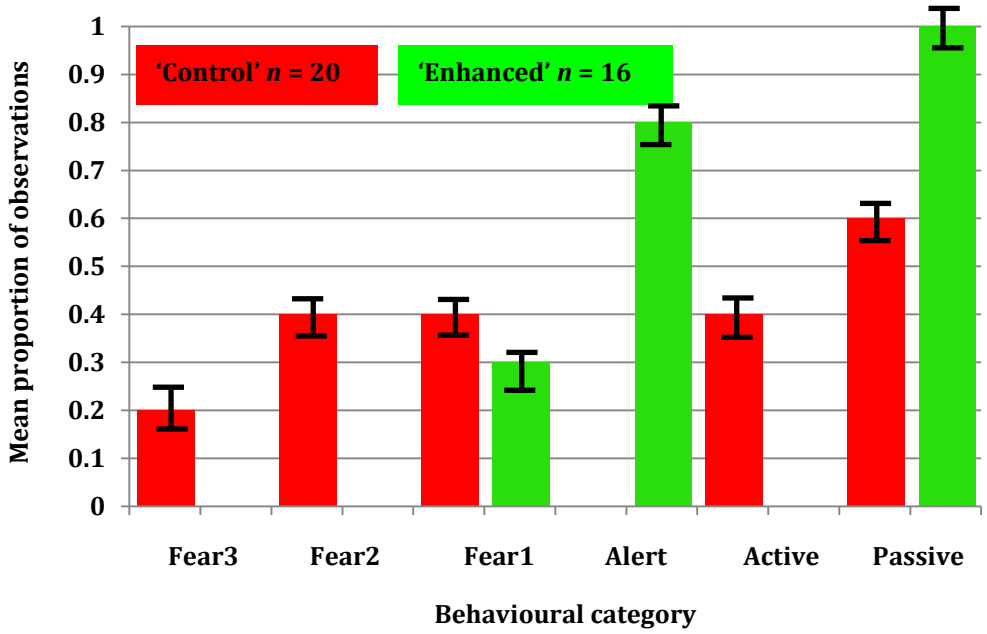
Kra calls were given by macaques in both Cohorts during capture test in week 3 and by 'Controls' but not 'Enhanced' during week 5. Coo calls were only given by 'Enhanced' group during the positive test, and they were found to call more in week 5 (week 3 total: 11, range: 1 – 5; week 5 total: 37, range 1 - 5). In the 'Enhanced' group during week 3 acclimatisation in positive and capture tests, vocalisations correlated with behavioural responses. During positive test, 'Coo' calls correlated postively with being in Contact (r = 0.71, p<0.05) and negatively with being Active (r = -0.78, p<0.05). Conversely during capture test, Kra calls correlated negatvely with in Contact (r = -0.76, p<0.05) and postively with being Active (r = 0.74, p<0.05). During tests in week 5, Coo calls given in positive tests were positively correlated with being Inactive (r = 0.71, p<0.05).

Vocalisations were not found to correlate with behaviours during human response tests in 'Control' animals in weeks 3 or 5.

e) Response to restraint for ECG and HDO recording

The behavioural responses of macaques during ECG and HDO recording are displayed in Figure 5.3.3 i.

Figure 5.3.3 i Mean behavioural responses of male macaques during ECG recording in 'Control' and 'Enhanced' groups during week 6 and week 8 respectively.



'Control' and 'Enhanced' males were not matched at ECG recording (e.g. 'Controls' recorded during week 6; 'Enhanced' week 8). Furthermore, 'Enhanced' animals were organised into different group sizes; 5, 3, 3, 5 configuration compared to groups of 5 males in the 'Control' study. The most obvious differences in behavioural responses between groups were that 'Enhanced' animals displayed Alert facial expressions and were Passive during recording. Macaques were not heard to vocalise during recording. Conversely, in 'Control' macaques 40% (n = 8) were heard emitting Kra (range: 4 – 6 calls), Wraagh (range: 1 – 2 calls) and Khreet Screach (range: 3 - 10) vocalisations. Vocalisations were given by active animals displaying Fear3 facial expression.

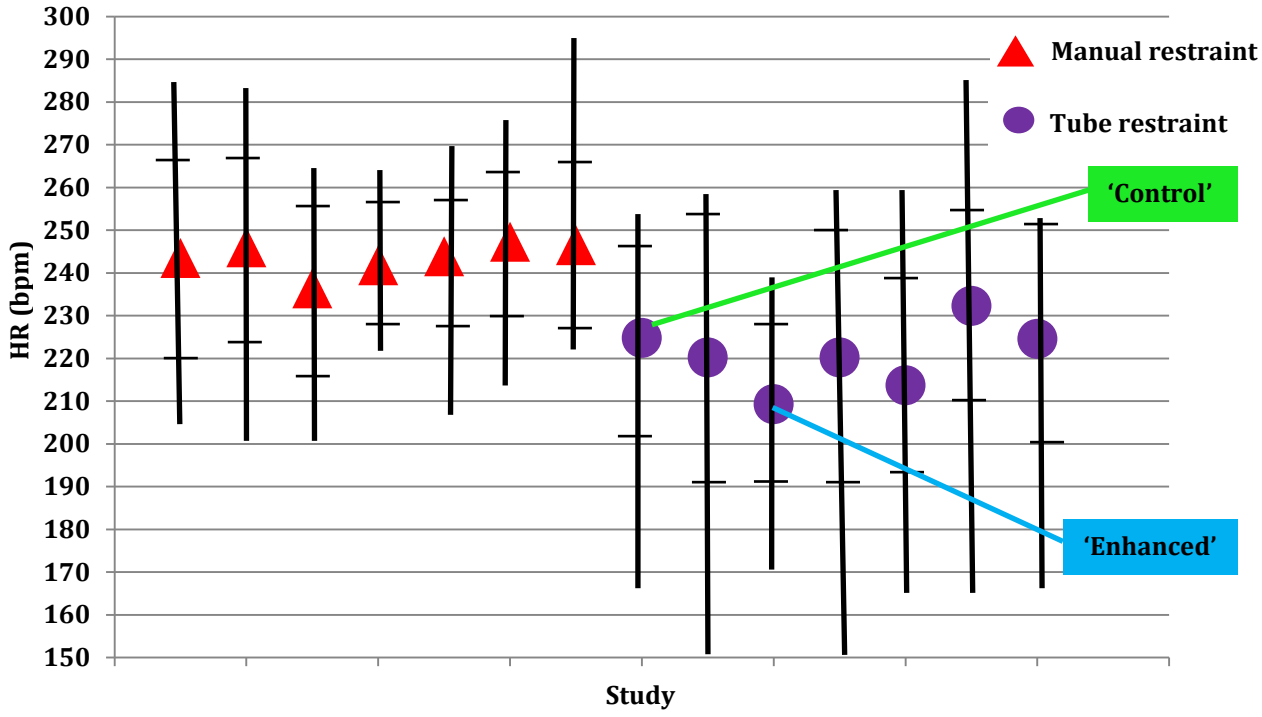
Behavioural responses during HDO recording are not shown owing to the additional confounds of time taken to record blood pressures in each group (Figure 5.3.3 k). The increased time and

number of attempts to record blood pressure measurements were related to being Active, vocalising and displaying Fear3 facial expressions in 'Control' animals. The additional movement and muscle tension associated with these behavioural responses cause artefacts in the trace (Appendix 1.5.3d) and recordings have to be repeated for accurate measurements.

5.3.4 Physiological responses

The effect of enhanced socialisation on cardiovascular parameters recorded at baseline are shown in Figures 5.3.3 j & k.

Figure 5.3.3 j Male macaque baseline heart rates from 14 studies: 7 using manual restraint and 7 using tube restraint.

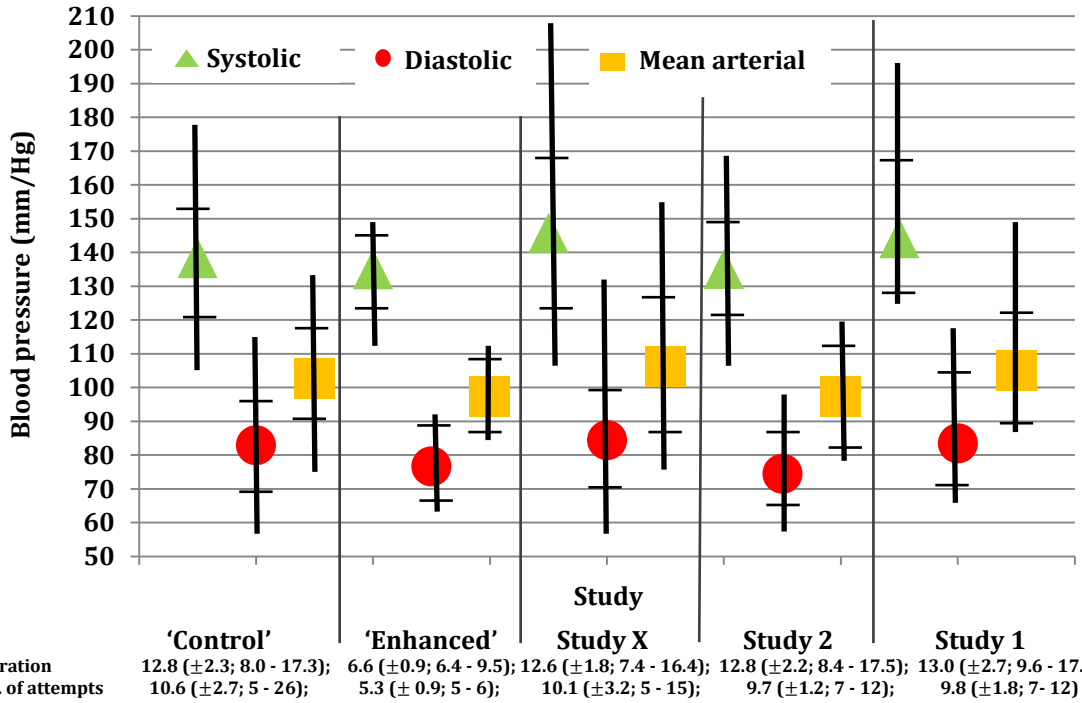


Male only data reported; Mean heart rate (bpm) ± SD (⊥); Observed range: minimum – maximum (|).

The last seven studies using manual restraint before the CASE sponsor changed to tube restraint are included on Figure 5.3.3 j for illustration. Heart rates of macaques manually restrained were higher than animals restrained in tubes (manual: 243.6 bpm±18.5; tube: 220.8 bpm ± 29.2). Furthermore the spread of data illustrated by the standard deviation and observed range was narrower in manually restrained animals compared to animals in tubes, indicating a ceiling effect (Chapter 2). 'Enhanced' macaques in tubes showed lowest mean heart rates (mean: 209.3 ± 25.0)

of all studies and displayed less between-animal variation than 'Controls' and the remaining five tube restrained studies reviewed.

Figure 5.3.3 k Blood pressures (systolic, diastolic and mean arterial) recorded by high definition oscillometry (HDO) in five studies using tube restraint.



Male only data reported. Blood pressure (mm/Hg) ± SD (⊥); Observed range: minimum – maximum (❙). Study X has not been reported elsewhere in the thesis. Study 2 & Study 1 are reported in Chapter 3. **Duration:** mean time taken to record ECG and 5 blood pressures (minutes; ±SD: Min – Max); **No. of attempts:** mean number of cuff inflations to record 5 successful blood pressures (±SD: Min – Max).

Blood pressures (e.g. systolic, diastolic, mean arterial) were lowest for 'Enhanced', but not significantly. Furthermore, 'Enhanced' displayed the least between-animal variation of all five studies reviewed as indicated by the observed range and spread around the central mean (± SD).

The effect of socialisation on cardiovascular parameters at baseline along with multiple predictors are displayed in Table 5.3.4.

Table 5.3.4 Regression model output: the effects of multiple predictors on cardiovascular parameters recorded from male macaques at baseline.

Cardiovascular (outcome) variable	Predictors	Model summary		Model parameters			Significance of model (ANOVA)	
		R^2	%	B	SE	β	*df*	F
HR	Constant Socialisation	 0.03*	 2.9	209.59 13.62	13.51 7.09	- 0.14*	1	2.09*
Overall model $R^2(3)$ = 0.03*; 2.9%								
Sys	Constant Socialisation	 0.03*	 3.1*	134.83 5.43	9.19 5.2	- 0.52*	1	2.70*
Overall model $R^2(4)$ = 0.03*; 3.1%								
Dia	Constant Socialisation	 0.05	 5.2*	87.92 -1.14	4.62 0.46	- -0.23*	1	6.18*
Overall model $R^2(1)$ = 0.05*; 5.2%								
MAP	Constant Time Socialisation	 0.04 0.08	 3.5* 4.4*	96.66 - 1.48 9.66	7.50 0.48 4.18	- - 0.38* -0.28*	2	4.78*
Overall model $R^2(2)$ = 0.08*; 7.9%								

Data given to two decimal places. R^2 (*df*) is a measure of how much variability in the cardiovascular (outcome) variable is accounted for by the predictors by SPSS to be included in the model. The significance of the model at predicting the outcome variable is determined in the model using ANOVA, *p<0.05, **p<0.01, ***p<0.001.

Socialisation was found to have the only significant, but small effect on three cardiovascular parameters out of all possible predictor variables entered into the model (e.g. HR: 2.9%; Sys: 3.1%; Dia: 5.2%), and it had the largest effect above time taken to record blood pressure in mean arterial pressure (MAP e.g. socialisation: 4.4%; time: 3.5%).

5.3.5 Summary of results: the effects of enhanced socialisation on welfare and scientific output

Macaques experiencing enhanced socialisation showed an increase in body weight overall with time spent in the unit whereas control animals showed weight loss or no overall gain during the five-week observation period where they were matched. Control animals lost weight (mean - 4.3%, p<0.05 males; - 1.8%, *ns* females) over the pretreatment period during baseline recording and showed no signs of stabilizing before onset of dose. Moreover, despite the time differences in scheduling baseline recording, male macaques that had been socialised did not lose body weight during the pretreatment period, although their rate of gain slowed. Body condition was found to track changes in body weight in both Cohorts and for both sexes. Consequently, control animals were either thin or very thin (e.g. 2.0; 1.5) at start of dosing in week 6, whereas socialised animals were lean or above (e.g. 2.5 or 3.0), and the majority of males were in ideal body condition (e.g. 3.0) at onset of dosing in week 9.

Incidence and severity of hair loss worsened during the pretreatment period (e.g. weeks 5 and 6) in controls. In contrast, enhanced animals showed improvements in pelage condition, which was

normal (e.g. 1; no hair loss) for most throughout observations, even when they went on to study in week 9. The higher incidence and severity of hair loss in controls may be related to the increased proportions of allo-grooming observed in the pre – handling observations during week 5. Furthermore, during pre – handling, socialised macaques spent a greater proportion of the observation time inactive and relaxed, displaying relaxed locomotion and playing compared to their matched counterparts in the control group.

Following handling both Cohorts were similar in their responses compared to their respective pre – handling behaviours. For example, they showed a narrower overall behavioural repertoire in the two-hour observation window, being more vigilant (e.g. watching the door), spending a greater proportion of the observation time Huddling, Embracing or in Contact whilst high up at the back of the pen. However, macaques experiencing enhanced socialisation seemed to show signs of recovery faster than controls; they spent less time being vigilant and there was a significant reduction in amount of time spent Huddling and increasing time Exploring in hour 2 compared to hour 1.

Macaques in both groups spent less time asleep before and after midnight following handling, and more time awake before midnight and active after midnight. Socialised macaques spent more time asleep after midnight during pre – handling observations than controls. They also appeared to have a less disrupted sleep pattern following handling in that they spent more time asleep and less time awake before and after midnight.

Indeed macaques in the enhanced group displayed fewer fearful behaviours during handling for weighing and physical examination in week 5 than matched controls. Furthermore, they were more similar in their behavioural responses, whereas control females displayed higher-grade fear responses (e.g. Fear3) and were more active during handling compared to their male counterparts.

Both groups showed signs of habituating to the presence of humans with acclimatisation, as indicated by their responses during three standardised tests. Compared to week 1 where no animals would approach the human observer, by week 5 all animals, in both Cohorts were

approaching and taking food from her hand in the positive test. The latency to approach decreased across testing sessions in weeks 3 and 5, although socialised animals took less time to approach than controls during testing in both those weeks. Macaques didn't habituate successfully to the capture test, never approaching to take food from the gloved hand, despite socialisation or with increasing time spent in the unit. The behavioural responses of macaques during 60 seconds following the 30-second human response test were more complicated to interpret. Macaques experiencing enhanced socialisation showed somewhat more positive changes with time compared to controls. For example, they were less often found to be in contact in both passive and positive tests over repeated sessions than controls; the latter showed no change in contact during the positive test and increased huddling in the passive test during week 5 compared to week 3. Overall, socialised animals showed comparatively fewer fearful responses in passive and positive tests (e.g. less time in contact or huddling) across testing sessions in weeks 3 and 5, indicating they were habituating to the presence of humans in the laboratory faster than controls exposed to normal unit procedures.

Although not matched by the time of ECG and HDO recording, socialised animals showed distinct behavioural differences during restraint for ECG when compared to controls or animals in Studies 1, 2 and 3 in Chapter 3. They were mainly Alert and Passive during recording, and this lack of activity and fearful responses was found to facilitate data collection, taking around half the time to record ECG and five repeated blood pressure measurements than in any of the five studies reviewed including controls. This was possible because every attempt to record blood pressure resulted in accurate measurement and was not confounded by movement artefacts in the trace caused by macaques vocalising or being active during recording.

Both heart rates and blood pressures showed considerable variation within and across reviewed studies. The last seven studies where manual restraint was used to record ECG traces before the CASE sponsor changed to tube restraint were included for illustration and partial comparison. Macaques in tubes had lower heart rates than macaques manually restrained, although owing to ceiling effects manually restrained animals showed less between-animal variation. Socialised male macaques restrained in tubes had the lowest heart rates overall, and demonstrated the least

between-animal variation out of seven studies. Similarly, out of five studies where HDO was used to record systolic, diastolic and mean arterial blood pressures, socialised macaques had the lowest blood pressures and least between animal variation. When using a multivariate approach to analysis, socialisation was found to have the largest or only effect on variation in heart rate or blood pressure, in contrast to acclimatisation time or habituation to tube restraint. Although these variations were significant they were small (range: 2.9 – 5.5%), and most of the variation in cardiovascular parameters could not be accounted for by my regression model.

5.4 Discussion

Juvenile and young adult macaques used in this study were in good health and not dosed with test article during my observations. The data reported in this Chapter are unlikely to be confounded by underlying pathology, and significant changes are likely to reflect changes in welfare state (e.g. Chapter 3). Both body weight and condition fell in controls compared to socialised animals over acclimatisation and during pretreatment procedures. Losses were not severe enough to be in line with recommended humane endpoints (Chapter 3), but the pattern of change over the pretreatment period is in agreement with my findings in Studies 1, 2 and 3 (Chapter 3). The observation of both loss in body weight and condition in controls may indicate they were coping less well with husbandry and regulated procedures during acclimatisation and the additional stress experienced was likely having a cost to juvenile animals, diverting resources away from growth (Chapter 3). The behavioural data discussed below also support this likelihood. However, the difference in food presentation three days a week (e.g. hand feeding) during socialisation sessions could account for this finding. Although measures were put in place to avoid bias relating to quantity of food delivered (e.g. weighing the same amount for each group) there's a possibility that by hand-feeding macaques I reduced monopolization of resources, allowing subordinates greater access to higher-value food items than their counterparts in the control group.

The increasing severity and incidence of alopecia during acclimatisation and pretreatment procedures along with higher levels of allo-grooming observed in controls during pre – handling further supports the notion that control animals were coping less well with the laboratory environment (Chapter 3). Indeed, higher levels of allo-grooming are thought to act as a stress

reduction mechanism (Schino *et al* 1988; Aureli *et al* 1989; Troisi *et al* 1991). The differences in behavioural responses during and after handling provide further evidence that 'Controls' weren't coping as well as the 'Enhanced'group. For example, socialised animals displayed significantly fewer fearful responses during and after handling, and appeared to show signs of recovery post - handling (e.g. reduction in huddling and increased locomotion in hour 2) compared to controls. What is more, significant reductions in anxiety and fearful response are consistently reported by other authors working with primates following a period of positive human interaction (e.g. Bayne *et al* 1993; Bloomsmith *et al* 1999; Baker 2004; Clay *et al* 2009; Manciocco *et al* 2009). Human responses tests were somewhat biased as socialised animals were tested in response to a familiar human compared to controls, who were less familiar with me; this could not be avoided as the test procedure was too time-consuming for care staff to perform. Nonetheless, both controls and socialised macaques were confident enough to take food from my hand and relatively quickly (e.g. $\leq$ 8 seconds) by week 5. However, neither group approached to take food from my gloved hand whilst I was wearing catching gear during the final response test even in week 5. This indicates that they continued to find capture stressful (e.g. Clarke *et al* 1993; 1995; Bowers *et al* 1998; Manuck *et al* 2009; Shively *et al* 2009) and had not habituated to it.

Heart rates were lower than those reported in Chapter 4, as a result of change in restraint and as expected they were considerably higher than those recorded for freely moving telemetered cynomolgus macaques (e.g. Strawn *et al* 1991; Authier *et al* 2007a & b; Bass *et al* 2009; Ishizaka *et al* 2009). There are no published references values with which to compare the data for macaque heart rates recorded by restraining animals in a tube. Blood pressures recorded using high definition oscillometry were in agreement with those published from conscious, restrained cynomolgus macaques (e.g. Schmelting *et al* 2008). However, they were higher than pressures reported by Chester *et al* (1992) from macaques restrained in tubes. This is likely to result from the greater opportunities for habituation that macaques experienced before baseline recording (e.g. 5 min/ 3xday/ week before recording; Chester *et al* 1992).

Using multivariate statistical techniques socialisation was found to have the main effect on variation in heart rates and blood pressures. Socialisation was associated with lower baseline

levels and a reduction of between-animal variation. Furthermore behavioural responses provide evidence macaques were less fearful during recording, and the lack of vocalisation, muscle tension and struggling during restraint facilitated faster data collection. Given this finding, macaques in receipt of enhanced socialisation appear to cope better with the laboratory environment and during procedures. Moreover, their baseline cardiovascular data were better quality, showing increased sensitivity and greater reliability (Chapter 2). This study provides quantitative data demonstrating the link between Refinement through socialisation with human care staff, enhanced welfare (e.g. reduction of fear responses) and improved quality of toxicological output.

6

General discussion and recommendations

"…refinement is never enough, and we should always seek further for reduction and if possible replacement."

Russell & Burch (1959), p 66.

It has been over 50 years since Russell and Burch (1959) published their seminal work, outlining the principles of humane science, the three Rs, *Replacement, Reduction and Refinement*, which now underpin the philosophies of laboratory animal science, regulatory guidelines and legislation controlling the use of animals in experiments (Chapter 1). They were also the first authors to make a case for the importance of animal welfare to scientific findings. It seems inconceivable now that these two concepts where never considered to be implicitly linked as the notion of *'good-welfare-and-good-science'* is universally made throughout the literature, guidance documents and statutory instruments (Chapter 2). Somewhat more surprising, with the rapid advancement of animal welfare (Section 2.2) as a branch of scientific endeavour in its own right, is that the link between the two has not been widely and systematically examined, particularly in non-human primates (Chapter 2). To this end, this thesis is the first body of work to systematically examine the link between welfare and quality of scientific outcomes in cynomolgus macaques (*Macaca fascicularis*) used for regulatory toxicology, by piggy-backing on commercial studies undertaken at a large contract research organisation.

6.1 Assessment of welfare of cynomolgus macaques

The assessment of macaque welfare is fundamental to Refinement and necessary to bring about improvements in care, housing and the conduct of procedures. Welfare is multidimensional in nature (Section 2.2), and multiple measures were recorded to produce an overall assessment of macaque welfare in the laboratory (Chapter 3). Body condition, alopecia, facial expression and vocalisation were found to be sensitive to changes in welfare state associated with handling and restraint, and change in group composition for study requirements. They are feasible for inclusion in to existing unit protocols that are comprehensive in their assessments of macaque health. Furthermore, fluctuations in body weight, and changes in activity budgets (e.g. patterns of activity, time spent in social contact), vigilance behaviours and position in the home pen provided useful indicators of whether macaques were becoming accustomed to the laboratory environment during acclimatisation, and in a stable state at baseline recording prior to start of commercial study. Stability is critical to ensuring reliability and repeatability of baseline data in the core battery of measures recorded by toxicologists (see below). What is more, baseline physiological parameters (e.g. heart rate, blood pressure, haematological and clinical chemistry analytes) were found to

correlate with macaque behavioural responses. Indeed, I found several positive and negative correlates of physiological and physical health parameters that co-varied with behaviours central to Poole's (1997) definition of a happy animal: '*one which is alert and busy (shows a wide behavioural repertoire), is able to rest in a relaxed manner, is confident (outward going and does not display fear towards trivial non-threatening stimuli) and does not show abnormal behaviour*'. These data support Poole's (1997) assertion that happiness is an inherent quality of both good welfare and good laboratory science.

6.2 Quality of scientific output

In Chapter 2, I outline that good toxicology is also based upon stable, normal, healthy, happy animals. Indeed, the quality of baseline biological data is critical for quantifying magnitude and direction of changes following chronic dosing with novel pharmaceuticals, and form the basis of risk assessments prior to human exposure. Three dimensions of quality of scientific output were defined including sensitivity, reliability and repeatability. This framework of terms was used to examine the effects of enhanced welfare on sensitivity and variation of baseline core battery data from macaques in regulatory studies.

6.3 Examining the link

a) Refining housing

Primates have an inherent need for social companionship; the presence of a compatible conspecific is enriching (Schapiro *et al* 1996) and enables primates to cope better with stressful laboratory events (Rennie & Buchanan-Smith 2006a). Over the last 10 - 15 years, group housing in toxicology has become commonplace (JWGR 2009), but the effects of changes in housing on macaques' biological data have not been examined.

Results of a review of historic data derived from macaques housed in different social conditions (Chapter 4) found housing accounted for a small amount (range 0.1-11%) of variation in baseline data. In order to quantify these changes a number of other animal- and unit- related covariates were defined (e.g. age, sex, acclimatisation time, group size, sham dosing etc.). This study highlighted the difficulties faced by toxicologists in standardizing macaque-related variables, in

stark contrast to rodent studies, where standardization of the animal model is universal and the main approach for reducing unwanted variation (chapter 2). Furthermore, this variation is often not considered as data across studies are rarely examined. Nonetheless, the extent to which standardization is effective in reducing variation is highly questionable and arguably at the expense of external validity (Chapter 2). Moreover, the confounding effects of numerous variables also meant that there were some difficulties in interpreting the hypothesized effects of welfare-positive, purpose-built group housing for macaques and quality of baseline data.

It appeared that sham dosing, undertaken to habituate animals to the procedure, had a negative effect on welfare and caused variation in Creatinine (CREAT), white blood cell (WBC), neutrophil (N) and lymphocyte (L) counts in macaques. This finding highlights the need to assess the impact of changes on welfare and quality of scientific output before incorporating them into standard operating procedures.

Heart rate was significantly lower in animals housed in purpose-built accommodation, even though they experienced more sham dosing. It was hypothesized that the design of purpose-built accommodation fosters more positive macaque-care staff interaction by affording greater opportunities for socialisation and avoiding hand capture, by use of a transport box. For example, the siting of multiple perches at various heights above the pen floor at the side, front and back of the pen enabled macaques to perform vertical flight if they felt threatened by human approach, as well as 'safe' places to observe human activity, and thus some control over human contact. In addition the wide bars at the front of the pen facilitated hand feeding and protected contact with care staff. The addition of a hatch in the door of the pen meant macaques could voluntarily run into transport boxes removing the need for hand capture. The effect of comparatively positive interactions may mitigate or buffer heart rate against the negative effects of sham dosing. Heart rate is particularly sensitive to psychological stress experienced during handling and restraint (Chapter 5).

b) Refining care-staff macaque interaction

Socialisation with humans in laboratories has been found to be beneficial to primates, and associated with reduction in anxiety-related behaviours (Chapter 5). Over a five-week programme of enhanced socialisation based around normal husbandry events and with additional focal sessions where human contact was paired with food, fear responses were significantly reduced and animals were more stable as they went on to study (e.g. body weight and body condition). Macaques were found to cope better with handling and restraint for husbandry procedures compared to controls, and had lower incidence and severity of alopecia.

Indeed enhanced socialisation was found to result in significantly lower heart rates and blood pressures in male macaques compared to controls. Furthermore, in a larger data review the socialised group had the lowest heart rates out of seven studies where tube restraint was used to record digital ECGs. Change in restraint was brought about by the CASE sponsor to improve quality of digitized ECGs and avoid poor trace quality associated with muscle tension produced by manual restraint (Appendix 1.2). Given that manual restraint is known to be stressful for primates, it was likely to be causing a ceiling effect, reducing the sensitivity of cardiovascular measures, by constraining further incremental responses with dosing. As a consequence of merely changing restraint, improved sensitivity through the removal of ceiling effects was observed, and inevitably the inter-animal variation increased. Enhanced socialisation greatly improved sensitivity by producing the lowest baseline heart rates out of six studies reviewed, more so than change in restraint alone and in combination with extended acclimatisation and habituation to tube device (Chapter 5). Furthermore, the socialised group were more homogeneous in their responses during baseline ECG and blood pressure recording. This enabled faster recording of blood pressures, and the time taken to perform ECG and HDO recording procedures were halved compared to controls.

Enhanced socialisation with care staff produced measurable improvements in animal welfare and quality of scientific output. Enhancing the sensitivity and reliability of cardiovascular measures by reducing the confounding effects of fear responses that accompany handling and restraint means toxicologists can more accurately quantify the magnitude and direction of change in heart rate and

blood pressure, with dosing, and establish a dose–dependent relationship – a critical component in safety and efficacy testing (Chapter 1).

This thesis has been the first systematic body of work to examine the link between welfare and quality of scientific output in cynomolgus macaques through Refinements to their housing, care and procedures. It provides specific information on improving their welfare, and for examining the link in toxicology studies, and many of these are encompassed within the recommendations below.

6.4 Recommendations

6.4.1 A framework of terms to examine quality of scientific output

Before we can begin to systematically examine and understand the link between Refinement, welfare and quality of scientific outcomes, we must come to an agreement on a framework of terms that would be useful to structure research and underlying methods for quantitatively tracking changes in quality of scientific output. I have proposed definitions for sensitivity, reliability and repeatability (Chapter 2) in relation to individual measures included in a core battery typically used for regulatory toxicology. This is only a starting point, and with renewed interest (e.g. Hartung 2009) in utilising historic data (Gottman *et al* 2001; Cronin 2005), driven by the need to reduce the number of animals used for research (Hoffman *et al* 2008) and in the development of alternatives (ICCVAM 1997), it is a matter of urgency that a consensus be reached in the scientific community about what we mean by quality and how to assess it; this may be achieved by setting up a working group. The main stakeholders in the working group should include toxicologists from contract research organisations, pharmaceutical companies and representatives from international regulatory bodies involved in the drug approval process (e.g. Europe: EMEA, Japan: PMDA, US: FDA). The overarching aims of the working group should include: (i) to define aspects of quality of toxicological studies in relation to their sensitivity, reliability and repeatability; (ii) to set out an assessment framework with which to objectively measure those characteristics; (iii) to seek inclusion of such definitions and the assessment framework in international guidelines on the conduct of regulatory studies and finally (iv) to set in motion a process for including such an assessment framework in the formal approval process of new pharmaceuticals undertaken by regulatory bodies .

6.4.2 Measures for an overall assessment of welfare

Assessment of aspects of physical health is both comprehensive and well-structured in the laboratory. However, the formal assessment of behaviour (Chapter 3), which are critical indicators of how macaques are faring (Chapter 2, 3 & 5) is less well developed. Monitoring behaviours has a distinct advantage over other measures; they are more visible and accessible for care staff, and they do not require complicated recording equipment or a personal licence for invasive procedures. Moreover, simple recording sheets could be included in husbandry logs for formal documentation of changes in behaviour over time and with laboratory events, and these could be related to other measures of physical health and physiology to give a more holistic view of welfare. The training of animal care staff is critical to this end. Indeed, in order to enable care staff to correctly and reliably record and interpret macaque behaviour, animal welfare scientists, primatologists etc., need to develop web - based resources that illustrate behaviours (e.g. facial expressions, vocalisations, postures etc.) and training courses that are of interest to and relevant for care staff. We all, however, have a responsibility to ensure that they are accessible to staff both in-house and externally.

In addition to formal assessment of behaviour, using a grading or scoring method for body condition and alopecia enabled a more sensitive way of tracking changes in prevalence and severity (Chapter 3). These can easily be included in weekly clinical examinations performed by technicians. What is more, they were found to be reliable with very little training and experience. Baseline physiological parameters (e.g. heart rate, blood pressure, haematological and clinical chemistry analytes) were also found to be sensitive indicators of welfare, and were used to assess the efficacy of Refinement. A process of benchmarking baseline physiological measures could be utilised more effectively to assess changes in relation to animal welfare. Indeed the CASE sponsor had some success with this approach, when Refining restraint for recording digital ECGs and HDO blood pressures (Chapter 5). To conclude this set of recommendations, I propose using multiple measures including behaviour, physical health and physiological indicators to give the most comprehensive understanding of welfare state; care staff should be trained and monitored to ensure they are accurately and reliably recording, and interpreting them.

6.4.3 Historic control data

Chapter 4 highlighted the numerous animal–specific (age, sex, age*sex interaction) and unit-specific (e.g. acclimatisation time, study preparation, sham dosing) predictors that account for variation in historic data. Traditionally, age and sex are used as partitioning factors in historic databases maintained for comparison to study data. Consideration should be given to use additional partitioning factors (e.g. age*sex interactions, acclimatisation time and study preparation etc.) for more accurate comparison.

6.4.4 Sham dosing

Increased frequency of sham dosing was found to have negative effects on macaque biological data, and accounted for between 0.3 - 15.8% variation at baseline, depending on the haematological and clinical chemistry analyte. Macaques do not appear to habituate to the procedure, and the process of sham dosing is likely to cause unwanted variation in baseline data. In light of these findings, I would recommend reviewing the necessity for sham dosing prior to study.

6.4.5 Housing macaques for regulatory toxicology

Group housing was found to have beneficial effects on heart rate; in part this was attributed to the design of purpose-built housing. It should be noted that macaque housing/cage layout/configuration can promote or become a barrier to positive interaction with human care staff, and this has important implications for both welfare and quality of scientific output. It is recommended that caging is designed to allow protected contact with care staff. This facilitates hand-feeding without entering the pen of newly acquired macaques, and in combination with an alternative to hand catching (e.g. training macaques to enter a transport box), would promote positive care staff-macaque relationships and reduce the potential for conflict.

6.4.6 Socialisation

The benefits of socialisation were numerous and included reduced fear responses and promotion of macaque welfare, enhanced sensitivity and reliability of cardiovascular measures, and faster data acquisition; it also had positive effects on care staff morale. What is more, staff had closer contact with animals, which facilitated the numerous daily checks. It is recommended (in addition

to good housing design) that socialisation is undertaken. It can easily fit around daily husbandry routines and staff should be encouraged to have positive interactions at every opportunity whilst they are performing their duties. This can be facilitated by hand-feeding part of the diet throughout the day.

6.4.7 Future research

This thesis has provided a framework for systematically examining the link between welfare and quality of science, a link that is now universally made, but with few quantitative supporting data. As with all good science, further work is needed to develop and refine this approach. Indeed, as our understanding of macaque welfare deepens with novel approaches to assessment, we can better target Refinements, which in turn has the potential to reduce the number of animals used, as we decrease unwanted variation and enhance the sensitivity, reliability and repeatability of toxicological measures – should scientific consensus consider them useful indicators of quality.

This thesis provides quantitative data on the strong link between welfare and quality of scientific outcomes. Whilst it is recognised that change is slow, and there are numerous barriers to the uptake of recommendations, we fundamentally require changes in attitude which are both within and out with the control of commercial laboratories. Improved welfare in the laboratory is of scientific and ethical importance for human patients and non-human research models alike, and it is hoped that these recommendations will be taken up, industry-wide, as a matter of urgency.

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Appendix

Appendix 1.1 Clinical pathology measures

Clinical pathology measures (includes haematology and clinical chemistry) are an important part of the safety assessment battery (Evans 1996; Weingand *et al* 1996; Hall 2007; Hall & Everds 2008), because they often provide information not detected by direct examination of organs and tissues, and indicate temporal associations with time and duration of dosing and observed clinical signs to allow interpretation of the biological significance of findings (Hall 2007). For example, they help to evaluate the effects on hepatic and renal function, oxygen carrying capacity of the blood, circulating cells of the immune system, haemostatic function, and systemic mineral and electrolyte homeostasis, which is useful for determining the biological significance of findings in toxicity and safety studies (Weingand *et al* 1996).

The core haematology tests recommended are total leukocyte (white blood cell) count, differential leukocyte count, erythrocyte (red blood cell) count, evaluation of red blood cell morphology, platelet (thrombocyte) count, haemoglobin concentration, haematocrit (or packed cell volume), mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration. Blood smears can be prepared for the determination of reticulocytes. In addition, prothrombin time and activated partial thromboplastin time and platelet count are the minimum recommended laboratory tests of haemostasis.

The core clinical chemistry tests recommended are glucose, urea nitrogen, creatinine, total protein, albumin, calculated globulin, calcium, sodium, potassium and total cholesterol. For hepatocellular evaluation, measurement of a minimum of two scientifically appropriate blood tests is recommended (e.g., alanine aminotransferase, aspartate aminotransferase and bile acids). For hepatobiliary evaluation, measurement of a minimum of two scientifically appropriate blood tests is recommended

(e.g. alkaline phosphatase, gamma glutamyltransferase, total bilirubin, or total bile acids). Table 1.1 gives an explanation of each of the haematology and clinical chemistry parameters. Hall (2007) gives guidance on the interpretation of clinical pathology measures commonly seen for macaques in regulatory toxicology studies.

Table 1.1 Clinical pathology: Haematology and clinical chemistry parameters and their physiological function.

	Abbr.	Parameter	Physiological function, uses and other considerations
Haematology	HB	Haemoglobin concentration	Found in erythrocytes and carries oxygen.[2] Helps to evaluate RBC mass[8] . Used as a measure of anaemia.[7]
	RBC	Red blood cell count	Known as erythrocytes. Largest number of cells in the blood. Erythrocyte survival time is approximately 85 - 100 days in macaques.[7] Effects on erythrocyte parameters typically reflect a change in the balance between RBC production and RBC loss[8] Changes in plasma volume through dehydration or volume expansion can indirectly affect erythrocyte parameters.[8]
	PCV	Packed cell volume	Measure of percentage of red blood cells in a sample of spun whole blood.[2] Used to estimate the degree of dehydration; the more dehydrated: higher PCV[7]
	RETA	Reticulocytes	Immature red blood cells. Develop and mature in bone marrow and circulate for a short period in blood before developing into mature red blood cells. Normal reticulocyte count is less than 1% in monkeys.[7]
	RABS	Absolute reticulocytes	As above.
	MCV	Mean cell volume	Measure of average red blood cell volume. MCV varies in cynomolgus monkeys depending on origin; larger in animals of China/Vietnam origin.[7]
	MCH	Mean corpuscular haemoglobin	The average mass of hemoglobin per red blood cell in a sample of blood.[2]
	MCHC	Mean cell haemoglobin concentration	Measure of the concentration of hemoglobin in a given volume of packed red blood cells. MCHC is lower in monkeys than other commonly used species in the laboratory.[7]
	HDW	Haemoglobin distribution width	Measure of the heterogeneity of the red cell hemoglobin concentration.
	RDW	Red cell distribution width	Measure of the variation of RBC width (volume).
	PLT	Platelets	Known as thrombocytes, they are important for blood coagulation (clotting), as they stimulate vasoconstriction and fibrin formation [7] Decreased platelet count is associated with prolonged bleeding e.g. from small wounds of venepuncture sites.[7]
	PCT	Platelet distribution	-
	MPV	Mean platelet volume	Measure of the average size of platelets found in blood.
	PDW	Platelet distribution width	Measure of variation - an indication of variation in platelet size which can be a sign of active platelet release
	PT	Prothrombin time	Prothrombin is a pre-cursor for thrombin[2]. Prothrombin activator splits the enzyme thrombin from Prothrombin. Thrombin is essential for clotting. Small changes are considered not to be biologically meaningful.[7]
	APTT	Activated partial thromboplastin time	A measure of coagulation mechanism by evaluating the intrinsic pathway. Responsible for converting prothrombin to thrombin, goes on to convert fibrinogen to fibrin[7]. Small changes are considered not to be biologically meaningful.[7]
	WBC	White blood cell count	Often termed leukocyte. Normally total white blood cell count is lower than RBC.[2] Important measure of immune function. Excited or frightened animals may have physiological leucocytosis (increased WBC count) due to endogenous catecholamine release, concurrently neutrophilia and lymphocytosis can occur[7]. The quantitative determination of total and differential (see next row) WBC are included[7]:
	N	Neutrophils	Most common WBC.[2] Granular leukocyte (granulocyte). Measure of immune function. Primary function is phagocytosis of small particles and integral to inflammation. Principal cell type found in peripheral blood[7]. Stress-induced leukocyte response may include mature neutrophila (no immature neutrophils).[7]
	L	Lymphocytes	Second most common WBC.[2] Non-granular leukocyte. Measure of immune function. Responsible for a wide variety of immune system functions. Principle cell type found in peripheral blood[7]. Stress-induced leukocyte response may include lymphopenia (decreased numbers).[7]
	M	Monocytes	Non-granular leukocyte. Measure of immune function. Primary function is phagocytosis and ingestion of large particles, processes antigens and present them to lymphocytes in a more antigenic form.[7] Usually only present in v. low no's.[7] Stress-induced leukocyte response may include monocytosis.[7]
	E	Eosinophils	Granulocyte.[2] Measure of immune function. Stress-induced leukocyte response may include eosinopenia (decreased numbers). [7] Present in low numbers.[7]
	B	Basophils	Granulocyte.[2] Usually only present in v. low numbers.[7] Measure of immune function.[2]
	LUC	Large unstained cells	Cells that have not taken up the stain during processing of blood and blood smears, therefore not red or white blood cells.

	Abbr.	Parameter	Physiological function, uses and other considerations
Clinical chemistry	AST	Aspartate aminotransferase	AST in blood derived from heart (cardiac muscle) (predominantly), liver, skeletal muscle.[2] Used in conjunction with ALT to identify site of tissue damage.[7]
	ALT	Alanine aminotransferase	ALT in blood derived from liver[2], highest activity in liver followed by cardiac muscle and kidney.[1] Can be high due to liver damage or liver cell damage may be markedly increased in animals that struggle during restraint thought to be through secondary iatrogenic muscle injury with handling or intramuscular injection of anaesthetic agents or sedative.[7]
	ALP	Alkaline phosphatase	Measurement represents joint enzyme activity – may be higher in juvenile growing animals.[1] Fairly non-specific enzymes, widely distributed in tissues difficult to interpret changes.[3]
	Gamma GT	Gammaglut-amyltransferase	Highest concentrations found in the kidney, pancreas and liver.[3] Used as an indicator for cholestasis.
	Na K Cl	Sodium Potassium Chloride	Electrolytes help to maintain fluid balance, pH, membrane potentials, muscular functions and nerve conduction etc. Sodium is the major cation in serum and the principal determinant of extracellular fluid volume.[7] Potassium is major intracellular cation, and is maintained in a narrow concentration range in serum, it is essential for muscle contraction.[7]
	Ca	Calcium	Involved in neuromuscular activity, bone formation, coagulation.[7]
	IN PHOS	Inorganic phosphorous	Essential for cell metabolism.[2] Similar functions to calcium, but more sensitive renal excretion and may serve as an indicator of renal function[6]. Levels vary with age; they are higher in very young animals.
	T PROT	Total protein	Plasma proteins consist of Albumins and Globulins.[2] Total protein is a measure of all the different proteins in plasma.[7] Involved in binding and transport of substances in the blood. Important for maintaining osmotic pressure and associated with immunity and disease resistance. Hyperproteinemia is associate with dehydration.[7]
	ALB	Albumin	Most abundant plasma protein.[2] Functions include binding and transportation of substances, maintaining osmotic pressure and preventing large fluctuations in PH by acting as a buffer.[4] Plasma proteins including Albumin indicate synthetic activity of the liver. Cellular damage reduces protein synthesis and the levels of plasma proteins decreases reflecting chronic liver damage.
	GLOB	Globulin	Heterogenic set of proteins with a number of functions (e.g. transport proteins, mediate inflammation, and immunoglobulins), important for immune function.[7]
	AG RATIO	Albumin/globulin	After hepatocellular damage (e.g. liver damage) reduction in Albumin accompanied by a relative increase in gamma GT producing obvious effects on the albumin/globulin ratio.[4]
	TOT CHOL	Total Cholesterol	Cholesterol is a precursor for steroid hormones.[2] Essential for cell membranes and a constituent of bile as it is required for the biosynthesis of bile acids. Endogenous it is produced by the liver.[4] Exogenous may be present in diet. Serum cholesterol is relatively stable [7]. Serum cholesterol and triglycerides may increase with age.[1]
	GLUC	Glucose	Serum glucose reflects numerous factors; stress and excitement may produce significant elevations of serum glucose.[1] Hyperglycaemia may be due to stress or feeding the animal prior to venipuncture.[7] The practice of fasting animals prior to venepuncture reportedly decreases the variability that accompanies post ingestion/digestion/intestinal absorption of glucose. [7]
	UREA	Urea	Urea nitrogen principally used to assess renal function.[1] Urea nitrogen is formed in the liver.[5] Urea and creatinine normally filtered from plasma by kidneys and therefore offers an indication of renal clearance.[7]
	T BILI	Total bilirubin	Bilirubin is a breakdown product of heam from RBC destruction.[2] The liver is responsible for metabolising spent RBCs and conjugating haem [4], making it a useful as an indicator of liver damage.[7]
	HCRE	Creatinine	Used as an indicator for renal function.[1] Formed in the muscle. Is a nonprotein nitrogenous waste material.[7] Creatinine kinase activity is highest in the skeletal muscle, cardiac muscle and brain[1]. It may be markedly increased in animals that struggle during restraint[7] as a result of secondary iatrogenic muscle injury with handling or intramuscular injection of anaesthetic agents or sedatives.
	TRIGS	Triglycerides	Fatty acid precursors, that have a large number of functions in the body e.g. in cell membranes.[4]

Abbr.: abbreviation; [1]Loeb 1989; [2] Frandson & Spurgeon 1992; [3]Evans 1996b; [4]Woodman 1996; [5]Stonard 1996; [6]Evans 1996c; [7]Hall 2007; [8]Hall & Everds 2008.

Appendix 1.2-1.5 gives an overview of the anatomy and physiology of the heart, and the principles of recording electrocardiograms (ECG) and blood pressure (BP) in cynomolgus macaques.

1.2 Anatomy and physiology of the heart

The heart and a system of vessels circulate blood around the body. The heart is a hollow muscular structure which both receives and pumps blood.

1.2.1 Anatomy

The heart is located in the thoracic cavity (chest), behind the sternum (breastbone) with its apex pointed down and slightly to the left (Figure 1.2.1). Figure 1.2.2 gives an anatomical view of the heart and associated structures. The heart is divided into left and right, each consisting of two chambers; an atrium, which receives blood through large veins, and a ventricle which pumps blood from the heart via arteries. The chambers are separated by valves which open and close in sequence preventing the back flow of blood. Specialized cardiac muscle cells generate electrical impulses that move through the heart muscle causing it to contract and force blood out into the arteries.

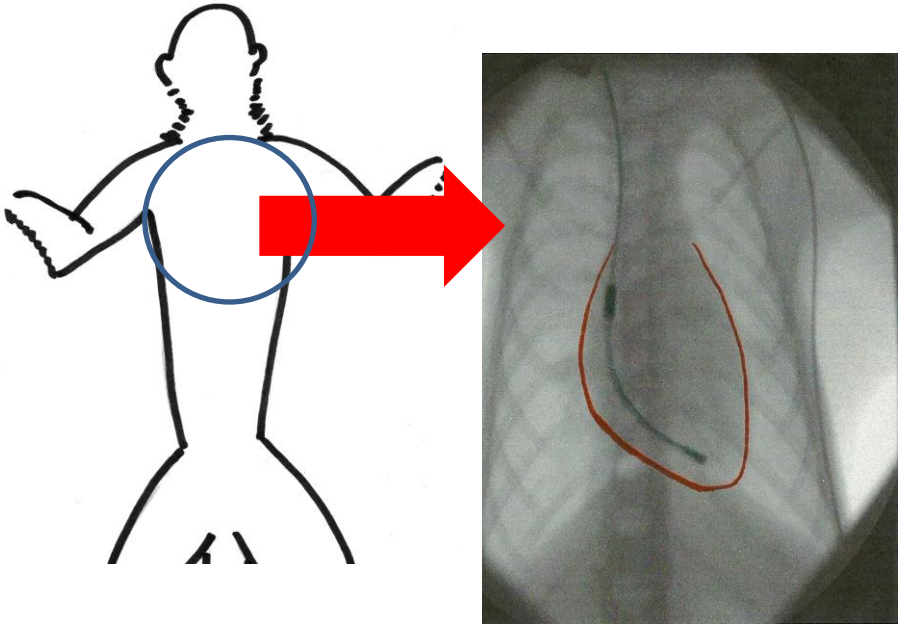
1.2.2 The cardiac cycle

The cardiac cycle refers to the sequence of events during one complete heart beat (Figures 1.2.3 & 1.2.4 a, b). The heart muscle cells continually undergo a cycle of depolarization and repolarization in a coordinated manner, enabling the heart muscle to contact and relax. The heart beat originates in the sinoatrial node (SA node) located in the right atrium, which is known as the "pacemaker" (Figure 1.2.2). The impulse from the SA node rapidly spreads throughout the atria causing them to contract (atrial systole). The atrioventricular (AV) node located in the septum between the atria detects the impulse generated by depolarization of the atria and conducts the impulse to the ventricular muscle via the AV bundle (bundle of His) and Purkinje fibres leading to ventricular depolarization and systole.

1.2.3 Regulation of the heart beat

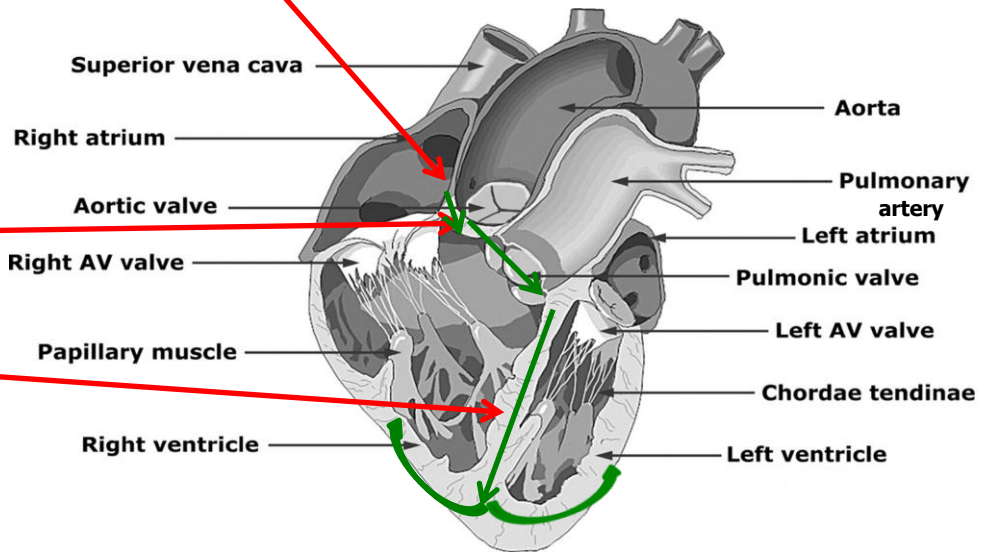
Intrinsic regulation of the heart beat is by the SA node, AV node, AV bundle and Purkinje fibres. Together they conduct impulses that are sufficient to keep the heart beating adequately without external nervous control. In addition, external innervations are regulated via the autonomic nervous system (ANS). Both heart rate (HR) and strength of contraction are influenced by the two branches of the ANS: parasympathetic and sympathetic, which act in a reciprocal fashion and maintain homeostasis. The parasympathetic nervous system acts on the heart via the vagus nerves to reduce heart rate, decreasing the force of muscular contraction and the rate of electrical impulses through the heart. Conversely, sympathetic stimulation (via stellate ganglia) increases heart rate.

Figure 1.2.1 Position of the heart in the thoracic cavity. View from the front. From Yao *et al* (2009).



Fluroscopic image taken from an anaesthetised cynomolgus macaque implanted with an intracardiac (IC) ECG lead. Note the position of the heart, with its apex skewed to the left. The outline of the heart is in red and the tip of the IC ECG lead is located in the right ventricle close to the apex of the heart.

Sinoatrial (SA) node – Located in the right atrium. Electrical impulses start here. Pacemaker of the heart.



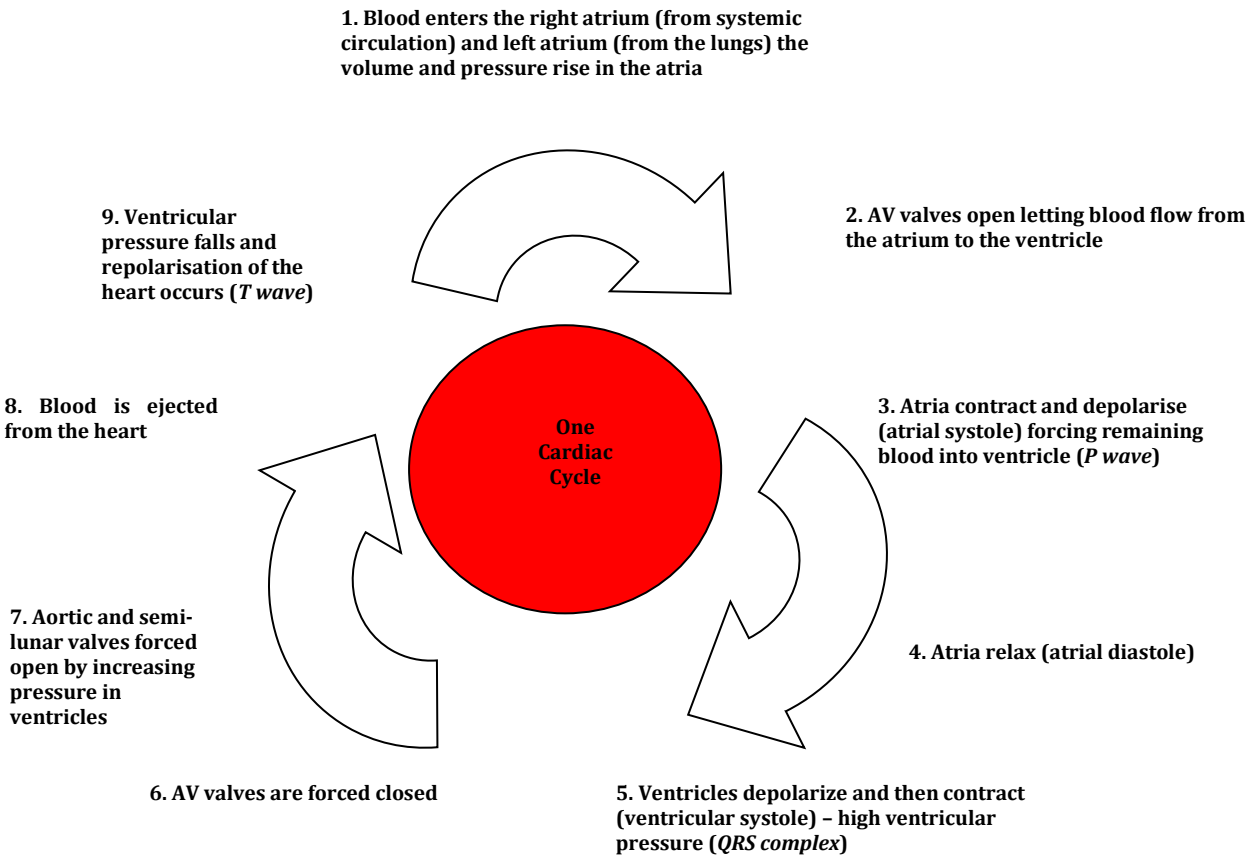
Atrioventricular (AV) node – Located in the septum between the atria. Detects impulse generated by SA node and conducts to AV Bundle and Purkinje fibres.

The wave of excitation travels along the fibres and causes the ventricles to contract from the apex upwards.

→ Denotes the direction of electrical conductivity across the atria and ventricles.

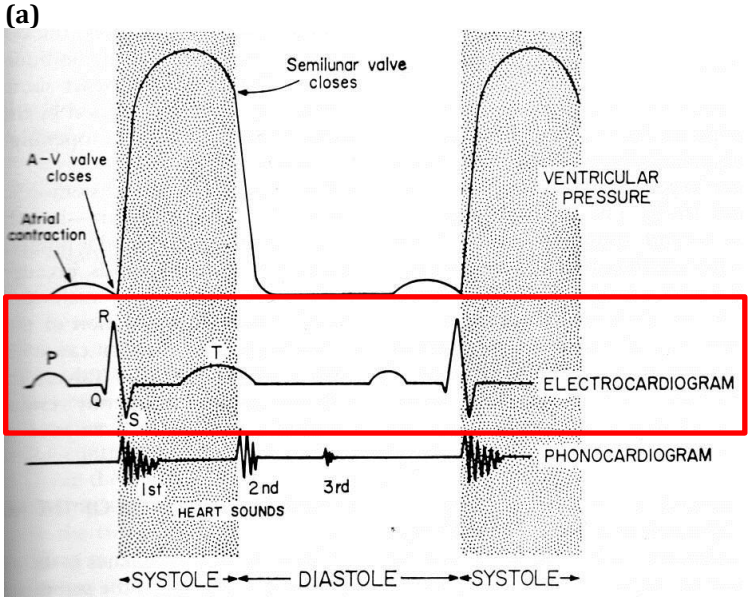
Figure 1.2.2 Anatomical overview of the heart with diagrammatic representation of electrical conductivity. From Donnelly (2008).

Figure 1.2.3 Events in a cardiac cycle which equate to one complete heart beat.

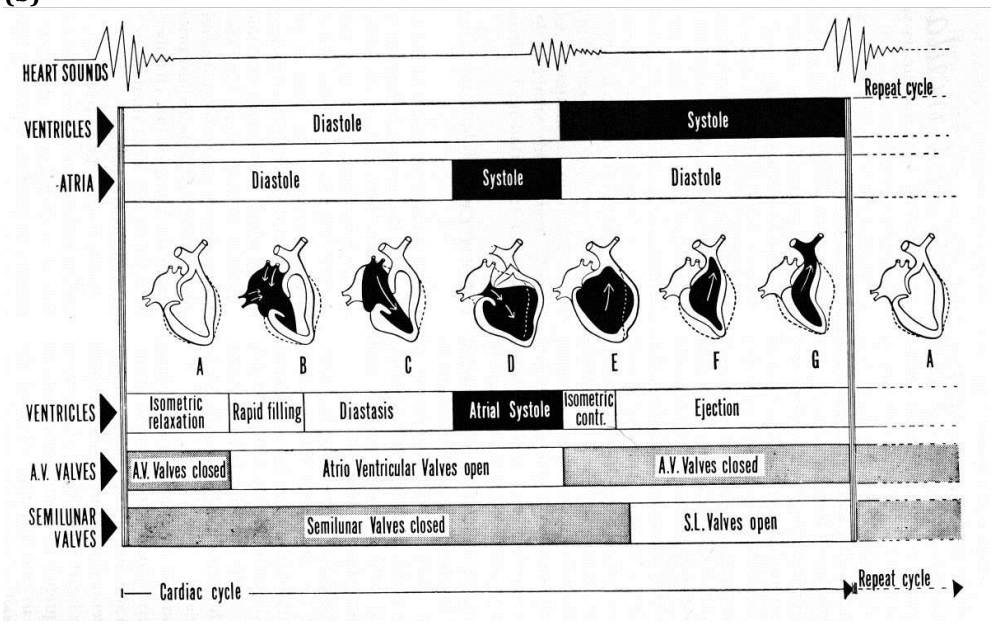


Figures 1.2.4 a & b The cardiac cycle illustrated in changes in cardiac events, ECG waveforms
(a) heart sounds (a, b) and blood pressure (a). From Frandson & Spurgeon (1992).

(a)



(b)



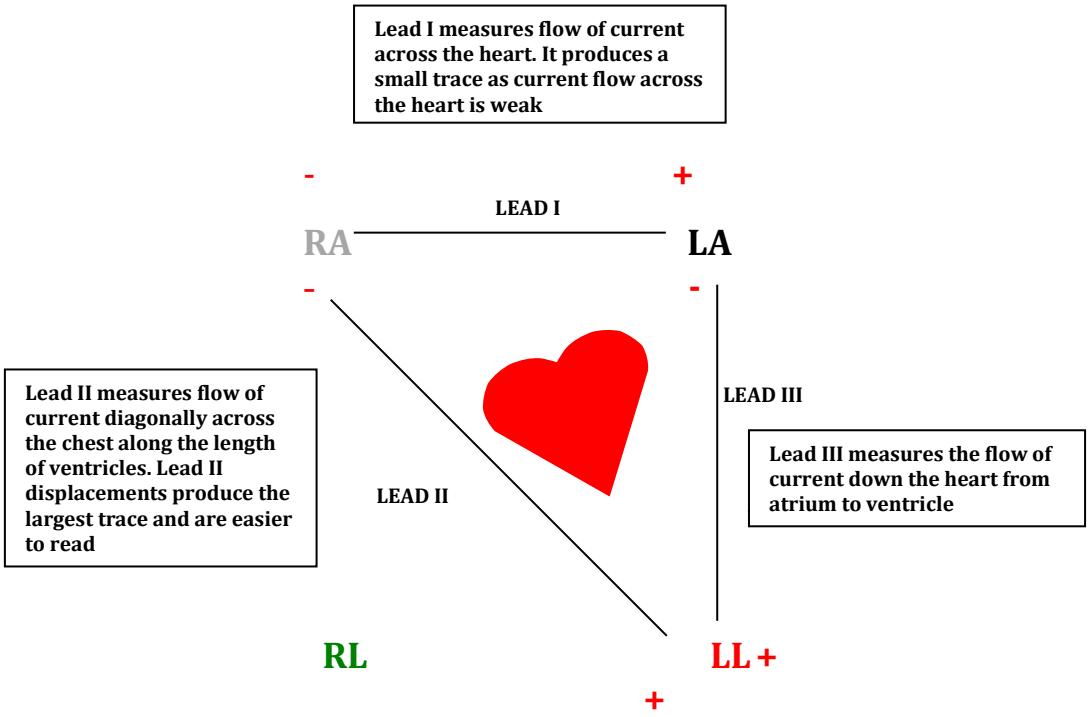
This sequence of events in the cardiac cycle produces a characteristic set of sounds (known as heart sounds), electrical activity (ECG) and pulsatile release of blood (pulse) causing changes in pressure (BP) and blood flow which can be measured directly and indirectly to assess cardiac and circulatory function (Figure 1.2.4 a & b).

1.3 Electrocardiograms (ECG)

Cardiac muscle displays electrical activity before, during and after contraction. Electrical currents spread through the heart and into the surrounding tissues and they can be recorded from body surfaces by a sensitive instrument known as the electrocardiograph (Frandson & Spurgeon 1992). The electrocardiograph is a sophisticated voltmeter that records changes in electrical potential (see Hamlin 2008). The electrical potentials between two leads, placed on the torso and limbs measure the electrical currents generated by the heart. A six-lead ECG recording is currently used for ECG measurement in cynomolgus macaques by the CASE sponsor. This includes 2 bipolar leads (Leads II and III) and unipolar leads; 3 augmented limb leads (aVL, aUR, AVF) and 1 chest lead (V_1V_2). Lead I measures the voltage difference between left and right side of the thorax, through the right (RA) and left (LA) chest electrodes. These electrodes are placed cranial to the heart on the left and right of the macaque's chest. Lead III measures the potential down the heart from the atrium to the ventricles through the left side of the chest (LA) and left leg (LL) electrodes. The left leg (LL) electrode is placed dorsal to the heart at the top of the left leg. Lead II measures the flow of current

running down the ventricles diagonally across the heart through the right side of the chest (RA) and left leg (LL) electrodes. The electrode placed at the top of the right leg (RL) is earthed. The arrangement of leads, in an equilateral triangle, is known as Einthoven's triangle; the heart lies at the centre of the lead configuration (Figure 1.3.1).

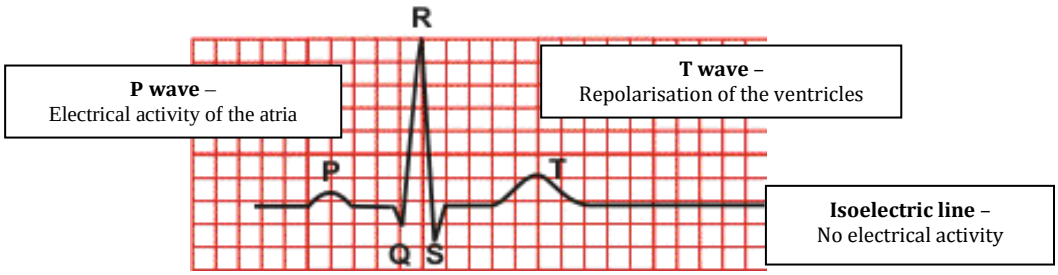
Figure 1.3.1 Standard lead placement for ECG recording by the CASE sponsor.



Lead placement: RA: Right side of the chest – approximately at the 4th intercostal space, 2cm from the sternal border (white lead); LA: Left side of the chest – approximately at the 4th intercostal space 2cm from the sternal border (black lead); LL: Left limb (red lead); RL: Right limb (green lead, earth/reference lead) – both are placed at the top of the leg. Current flows from negative to positive.

The graphic record that is produced by fluctuations in the electrocardiograph is known as the electrocardiogram (ECG) (Figures 1.3.2 & 1.3.3). Major waves on the ECG; P, Q, R, S and T, originate from electrical events in the cardiac cycle (Figures 1.3.2 & 1.3.3).

Figure 1.3.2 Waves of the electrocardiogram and electrical events occurring in the heart during a cardiac cycle.



Wave	Description of events
P wave	Spread of electrical activity from the SA node through the atria, creating depolarization. Associated with *ATRIAL SYSTOLE.*
P-R segment	Delay at the AV node.
QRS waves	Spread of the electrical impulse over the AV node and the ventricles and leads to depolarization. Associated with *VENTRICULAR SYSTOLE.*
T wave	Repolarisation of the ventricles. Marks the end of ventricular systole.
Isoelectric line	No electrical activity.

Both the height and width of the waves as well as the interval between them in the electrocardiogram can be measured and give an indication of cardiac function. The ECG is recorded on to paper (or in the case of digitised ECGs a virtual trace); calibrated to run at a constant speed, the distance on the trace is a measure of time (milliseconds - mS) and vertical displacement of the waves gives the size of the electrical potential (milliVolts – mV).

1.3.1 ECG measures of interest in regulatory toxicology

ECG waves are analysed to characterise effects on cardiac function that may translate to morbidity or mortality in humans during *in vivo* testing of pharmaceuticals (Hamlin 2008; see Chapter 1). In particular the periodicity of the heart (R-R interval), rhythm (sequences of depolarization and repolarization), atrioventricular conduction (PQ interval), duration of ventricular conduction (QRS duration) and ventricular repolarization (QT interval and QT interval corrected for heart rate) are considered useful (Hamlin 2008) (Figures 1.3.2 & 1.3.3), but not always required depending on the test article.

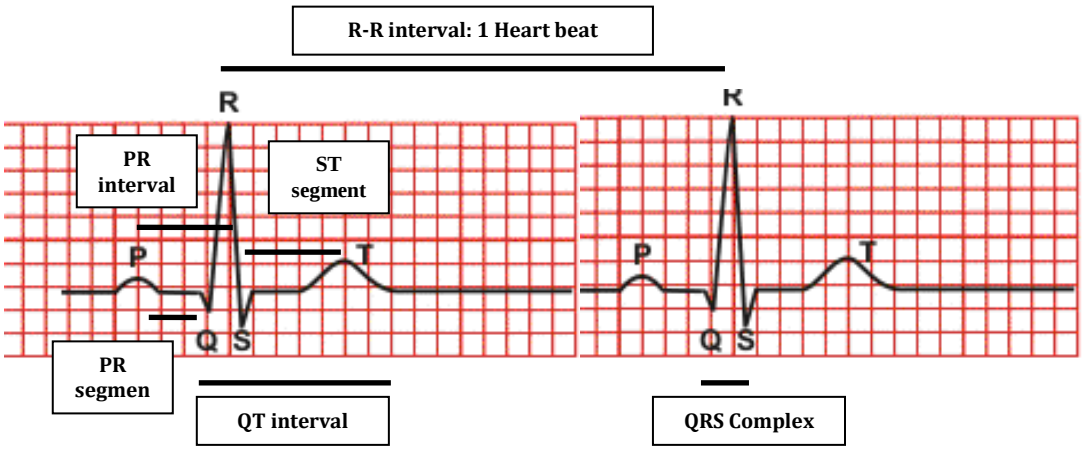


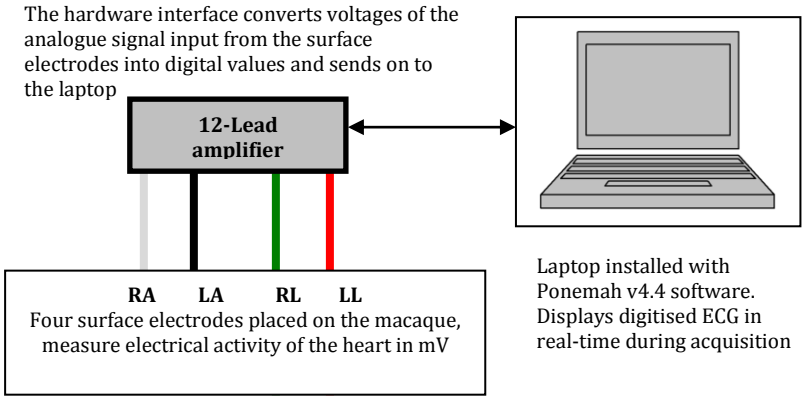
Figure 1.3.3 Waveform measurement in the ECG.

1.3.2 Digitized ECGs at the CASE sponsor

1.3.2a ECG machine

Ponemah version 4.4 physiological platform from Data Service International, Valley View, Ohio was used to acquire ECG data from macaques. The Ponemah system includes a portable ECG amplifier (ACQ-16 Parallel Legacy) with an analogue ECG board and an analogue to digital interface that converts the analogue signal (voltage) measured as a result of electrical conductivity in the heart from the surface electrodes to a digital wave form. The amplifier is connected to a portable laptop installed with the Ponemah software that allows continuous, digitised ECG data acquisition and analysis (Figure 1.3.4). Prior to acquisition the system is calibrated and attributes programmed by the ECG analyst.

Figure 1.3.4 Diagrammatic overview of Ponemah for the acquisition and analysis of digital ECGs.



1.3.2b ECG data acquisition

At least 30 seconds of good visible data was acquired from each macaque.

1.3.2c ECG data analysis

All ECGs were reviewed by ECG analysts. The ECG trace when played back in real time with beat-to-beat logging (epoch mode) was checked for the presence of arrhythmias. At least six consecutive wave complexes (PQRST; 7 R-R peaks; see Hamlin *et al* 2004) that best represent the data were selected for analysis. ECG parameters were measured from Lead II (Hamlin 2008). The analysts place tick marks at the beginning of the P, Q, R and S wave, and end of the T wave (Figure 1.3.5) to enable calculation of ECG parameters by the Ponemah software (Table 1.3.1). It took approximately 2-3 minutes to review each trace. Summary ECG data were exported directly to an Excel file. Parameters are reported for individuals, group means ± standard deviations, and observed range (min-max).

Table 1.3.1 ECG parameters and units of measurements used at the CASE sponsor, study dependent.

ECG parameter	Description	Unit of measurement
RR - I	Time interval from one R wave to the next.	Milliseconds (mSec)
QRS	Time interval of QRS complex from Q wave to S wave.	mSec
QT - I	QT interval measured from the Q wave to the end of the following T wave. Ventricular systole, this will vary with heart rate and must be corrected using a formula:	mSec
Corrected QT (QTc) using Bazett's or Fridericia's equations *	Corrected QT interval: Bazett's method: Computed QT interval divided by the square root of the RR-I. Average RR-I is used. Fridericia's method: Computed as the QT interval divided by the cube root of the RR-I. Average RR-I is used.	mSec
PR-I	PR interval measured from the start of the P wave to the beginning of the Q wave.	mSec
Heart Rate	Reciprocal of RR-I for the cardiac cycle multiplied by 60.	Beats per minute (BPM)

*** The equation used to calculate QT interval corrected for heart rate depends on the individual study.**

Figure 1.3.5 Example of ECG trace from Ponemah with tick marks identifying waveforms (P, R and T). Beat-to-beat review mode. Readings are taken from lead II.

1.3.2d Trace quality

High quality ECG traces are essential for precise evaluation of waveforms and are an important component in the battery of measures during preclinical drug testing (Guth *et a*l 2009; Chapter 1). Yet there are many factors that affect trace quality and therefore parameters; environmental (Chapter 4 & 5), physiological (Chapter 3), recording equipment etc. (reviewed in Hamlin 2005; Guth *et al* 2009; Authier *et al* 2010). Poor quality caused by muscle tension (Figure 1.3.6) and movement (Champeroux et al 2009; Figures 1.3.7 a - d) resulted in change of restraint of macaques at the CASE sponsor from manual restraint by technician (Chapter 4) to tube restraint (Chapter 5; Kelly 2009). Disturbances such as these hamper the reviewer's ability to accurately determine waveforms and subsequent ECG parameters and may obscure toxicological effects. High heart rates as a result of stress or arousal in response to environmental stimuli (Guth *et al* 2009; Authier *et al* 2010) also create problems for the measurement of waveforms as heart rate increases the tendency for P and T waves to fuse from consecutive cardiac cycles.

Figure 1.3.6 Muscle tension with manual restraint creates a 50Hz 'spike' of electrical interference in the ECG trace.

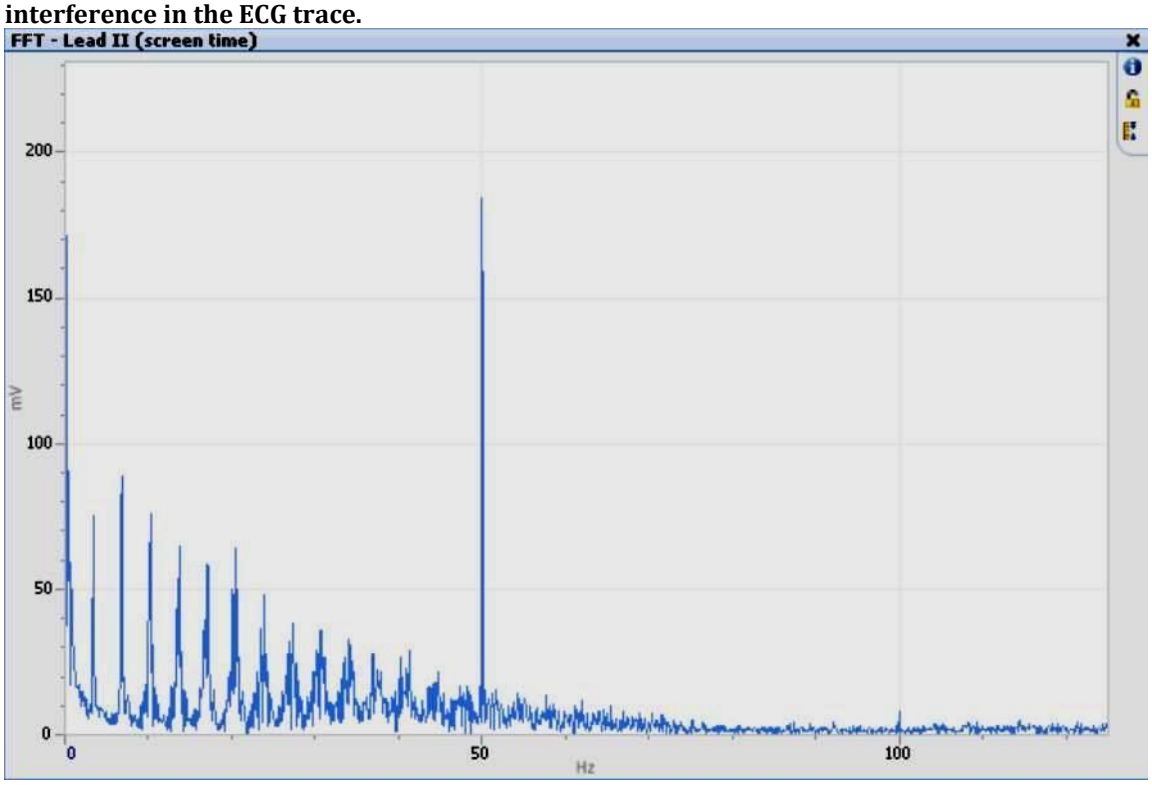
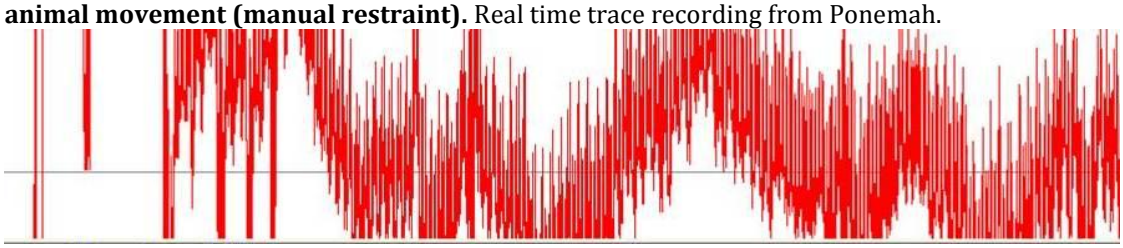
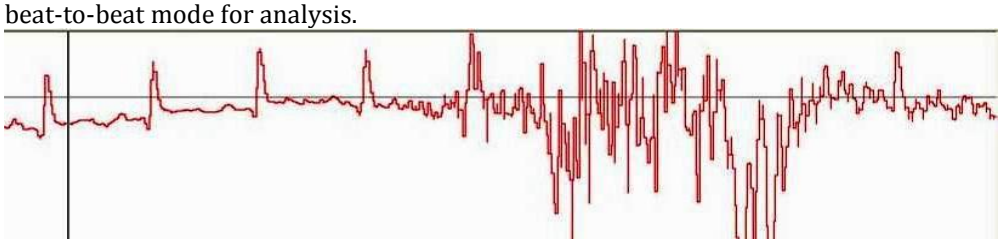


Figure 1.3.7a Artefacts in ECG trace from a macaque as a consequence of muscle tension and animal movement (manual restraint). Real time trace recording from Ponemah.



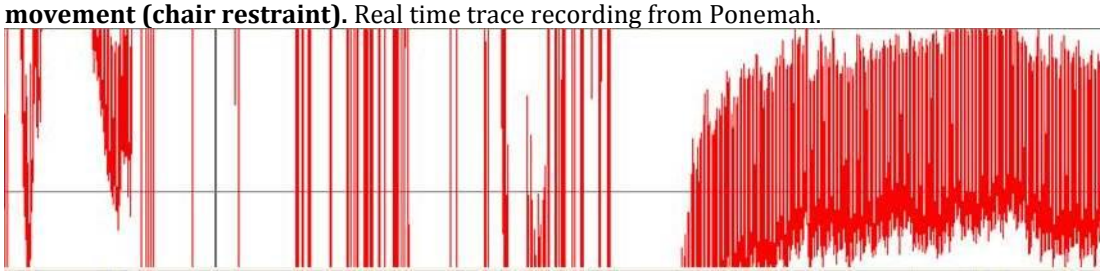
Sustained animal movement

Figure 1.3.7b Artefacts in ECG trace from a macaque as a consequence of muscle tension (manual restraint); baseline is not clean and difficult to identify all waveforms. Trace seen in beat-to-beat mode for analysis.



Muscle tension affects waveform morphology on the trace.

Figure 1.3.7c Artefacts in ECG trace from a macaque as a consequence of sustained movement (chair restraint). Real time trace recording from Ponemah.

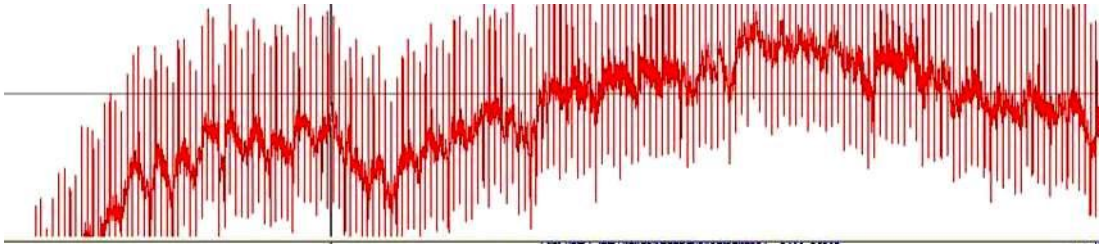


Sustained animal movement; interrupts the trace and prolongs acquisition to get an uninterrupted 30 second recording.

Figure 1.3.7d Artefacts in ECG trace from a macaque as a consequence of muscle tension (chair restraint), albeit reduced in comparison to Figure 1.3.7b. Trace seen in beat-to-beat mode for analysis.

It is difficult to identify all the waveforms of interest, particularly P and T waves.

Figure 1.3.7e A 'clean' trace from a macaque with reduced muscle tension and minimal animal movement (tube restraint). Real time trace recording from Ponemah.



Uninterrupted trace with no visible artefacts (though wandering baseline).

Figure 1.3.7f A 'clean' trace from a macaque with reduced muscle tension and minimal animal movement (tube restraint). Trace seen in beat-to-beat mode for analysis.

Normal waveform morphology. All wave forms are distinguishable for analysis.

All are lead II traces. From Kelly (2009).

1.4 Blood pressure definition and determinants

Blood pressure may be defined as the pressure blood exerts against the vessel walls (Frandson & Spurgeon 1992). In healthy animals, blood pressure is maintained by homeostasis between narrow ranges to ensure tissue perfusion and thereby oxygenation, delivery of nutrients and removal of waste products (Egner 2007).

The initial pressure, produced by contraction of the ventricles, is known as systolic pressure (SYS). Blood is forced into the large elastic arteries causing their walls to stretch and as a consequence they dilate. When the ventricles relax, closure of the semilunar valves prevents the return of the blood from the arteries to the heart, and the small arterioles impede the flow of the blood to the capillaries. Pressure exerted by the elastic walls of the arteries maintains blood pressure (diastolic pressure) (DIA) within the arteries and keeps blood flowing smoothly into the capillaries while the ventricles are relaxed.

Arterial blood pressure (BP) is influenced by factors that affect cardiac output (CO), total peripheral resistance (TPR) such as vascular factors e.g. vasodilation and vasoconstriction and hemorheological factors e.g. blood viscosity (Table 1.4.1; Egner 2007). Arterial pressure results from complex interactions between cardiac output and total peripheral resistance:

$$\mathbf{BP = CO \times TPR}$$

Cardiac output and total peripheral resistance can change independently of each other. Cardiac output - the volume of blood expelled per minute by the heart depends on the heart rate (HR) and the stroke volume (SV):

$$\mathbf{CO = HR \times SV}$$

Where stroke volume is related to diastolic filling volume and contractility of the ventricles. The higher the ventricular filling rate or the greater the strength of ventricular contraction, the higher the stroke volume.

Total peripheral resistance (TPR) is determined by the diameter of the blood vessels. With vasoconstriction, TPR increases the resistance against which the heart must pump (known as

afterload). During vasodilation TPR is decreased. Blood viscosity can also influence blood pressure which may be determined by the red blood cell count, red cell aggregation, plasma viscosity and temperature.

Table 1.4.1 Interrelated effects of cardiac output and blood pressure.

Cardiac output = Heart rate X Stroke volume:		
	Preload	**Contractility**
Increases in response to stress – catecholamines.	Increases due to sodium and water retention, fluid therapy etc.	Increases due to catecholamines, drugs and cardiac disease.
Increases in response to cardiac disease.	Decreases due to high heart rate.	Increases with increased contractility and increased preload.
Increases in response to systemic disease.		Increases due to decreased afterload.
Increases in response to central effects on the vasomotor centre.		Increases with certain drugs.
Increases in response to drugs.		Decreases with certain drugs.

Afterload: Resistance against which the heart must pump; **Preload:** Pressure and volume at which the blood enters the heart.

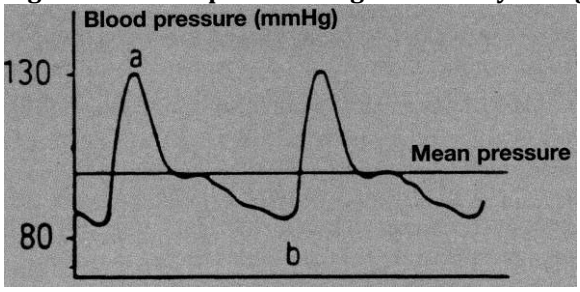
Adapted from Egner (2007).

1.4.1 Systolic (SYS), diastolic (DIA) and mean arterial pressure (MAP)

Blood entering the left side of the heart reaches the left atrium under low pressure. Blood flows passively into the ventricle during diastole. As diastole comes to an end, the atrium contracts, emptying the rest of the blood into the ventricle, but this does not lead to a significant rise in intraventricular pressure. However, during systole the pressure in the ventricle begins to increase and blood is ejected through the aortic valve into the aorta and out into the greater circulation. This systolic arterial pressure (SYS pulse) is produced with each contraction of the heart (Figure 1.4.1) and is determined by the stroke volume of the left ventricle, the speed of blood ejection (ejection velocity) and elasticity of the aorta. Diastolic arterial pressure (DIA) is determined by the duration of diastole, the volume of blood circulating and the degree of arterial elasticity. The mean arterial pressure (MAP) is the mean pressure throughout the duration of the stroke interval and can be calculated if SYS and DIA arterial pressures are known:

$$\mathbf{MAP = DIA + 1/3 \times (SYS - DIA)}$$

Figure 1.4.1 The pulse wave generated by changes in blood pressure.



a is systolic pressure (SYS); b is diastolic pressure (DIA). From Egner (2007).

1.5 Blood pressure measurement

The pulse wave is the key determinant of blood pressure (Egner 2007). Pulse waves are generated by pressure and volume changes as the heart ejects blood from left ventricle (systole) and fills (diastole). Systemic arterial blood pressure can be assessed either directly or indirectly. Direct measurements are obtained invasively by inserting an electronic pressure sensor into the artery. Indirect measurements are obtained non-invasively using external devices to occlude the blood flow in the limbs or tail (See Mitchell *et al* 2008; Schmelting *et al* 2008).

1.5.1 Indirect non-invasive blood pressure measurement

Blood pressure is measured indirectly using cuff methods, where the cuff is inflated to occlude arterial flow, and gradually deflated to allow flow to resume (Figure 1.5.1). The pressure indicated when blood first starts to flow through the artery as the pressure cuff is released is known as systolic pressure (SYS). Once the cuff no longer has an arterial flow, this is arterial pressure. Pressure is detected by transducers/sensors in the cuff. Accurate measurements are therefore dependent on the cuff size and fitting, and these should be selected according to the circumference of the target limb. If the cuff is too wide, the measurements may be falsely low, too narrow and the measurements are falsely high (Erhardt *et al* 2007).

Non-invasive indirect blood pressure evaluation can be achieved by Doppler ultrasound, oscillometry, high definition oscillometry (HDO) or pulse oximetry (see Erhardt *et al* 2007 for discussion of each). The CASE sponsor uses high definition oscillometry (HDO) to determine indirect blood pressure measurement in cynomolgus macaques. Blood pressure is conventionally measured in millimetres of mercury (mmHg).

1.5.2 High definition oscillometry (HDO)

High definition oscillometry has recently been validated in the cynomolgus macaque for use in regulatory toxicology (Mitchell *et al* 2008; Schmelting *et al* 2008). Values for systolic (SYS), diastolic (DIA), mean arterial pressure (MAP) showed good correlation with gold standard method for determination in chronically telemetered cynomolgus monkeys (Mitchell *et al* 2008). It

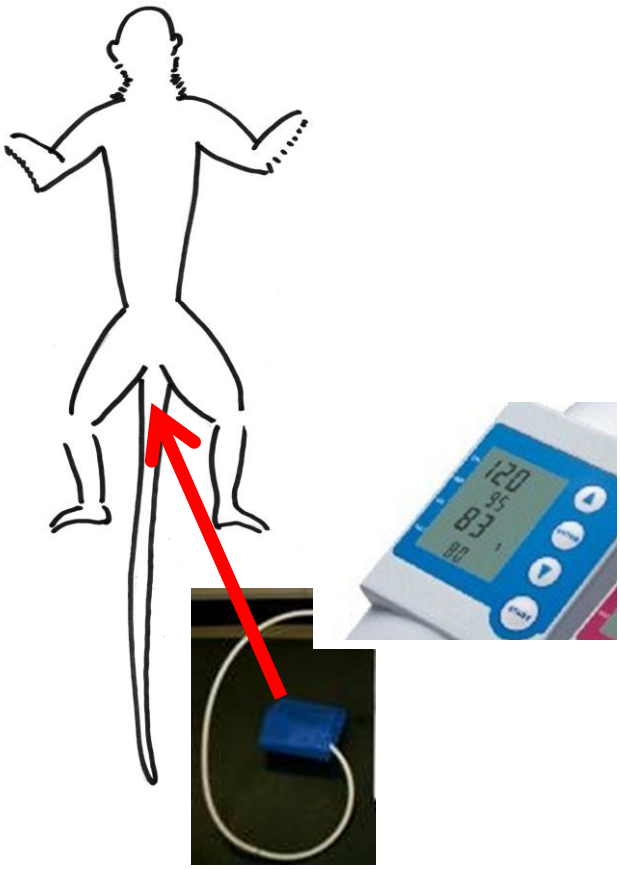
produced reliable and accurate blood pressure in animals that were manually restrained or sedated (Mitchell *et al* 2008; Schmelting *et al* 2008).

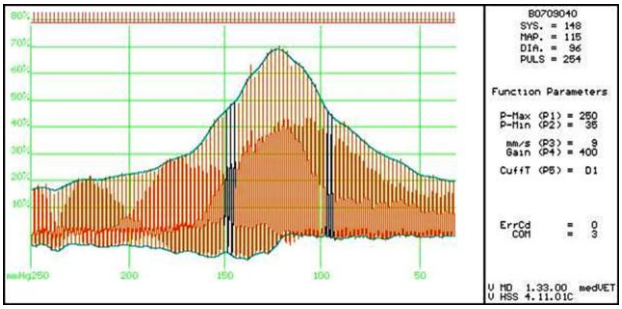
Table 1.5.1 Components of high definition oscillometry (HDO) blood pressure measurement system and acquisition attributes.

Term	Description
Cuff	Pneumatic cuff placed around the animal's arm, leg or tail inflates to occlude the artery.
Inflation/ deflation rate	Deflation rate: speed of cuff deflation in mmHg per minute. A minimum rate is the rate at which one heart beat per second can be measured.
Gain	Amplifies the pressure amplitude signal. Higher gains are needed for weak, low amplitudes and fast pulses.
Manometer	Pressure measuring instrument.
HDO	Measurement of the pulse wave-related oscillations of the arterial wall. This is visible in real-time on a laptop (Figure 1.5.1 and 1.5.2). A special algorithm is used for detecting amplitudes of systolic, diastolic and mean arterial pressure. This system is very sensitive and quick, measuring entire pressure ranges up to 300 mm Hg within milliseconds.
Processor	Responsible for the calculation and performance of the electronic device. The HDO processor has a 32-bit capacity allowing it to perform calculations in less than 1 microsecond time lag. This permits real time valve control and adjustment increasing accuracy of the system.
Real time analysis	Real-time analysis allows signal amplification and resolution. This can differentiate between amplitudes of systolic, diastolic and artefacts. The blood pressure trace, displayed on a laptop screen is visually appraised for artefacts or arrhythmias by the technician.
Sampling rate	Slice by slice signal resolution as in computed tomography.
Valve characteristics	Valves are used to regulate the cuff deflation rate. When used to measure high or low pressure ranges the valve must be programmable for real-time analysis allowing for accurate measurements.

Adapted from Erhardt *et al* (2007).

Figure 1.5.1 Diagrammatic representation of blood pressure measurement using HDO in macaques by the CASE sponsor.





The macaque is restrained in a tube (Chapters 3 & 5), the cuff is placed at the base of the tail and attached to the blood pressure machine that inflates and deflates the cuff. The microprocessor determines blood pressure measurements from the digital trace and displayed on the laptop.

1.5.3 High definition oscillometry blood pressure measurement

1.5.3a Blood pressure machine

A modified pneumatic cuff is placed around the base of the tail of the macaque[1]. When the cuff inflates it occludes the coccygeal artery. Re-entry of blood into the constricted space causes the artery wall to vibrate. These oscillations travel through the soft tissues to the surface of the tail, where they are detected by sensors in the cuff. The oscillations are characteristic of the systolic blood pressure, diastolic pressure and mean arterial pressure.

The pneumatic cuff is automatically inflated and deflated by the system control unit at a defined rate; approximately 40 second duration for 1 inflation and subsequent cuff deflation. The systolic and diastolic pressure valves are computed during the deflation phase. The cuff and sensor form a single measuring unit. Multiple readings are taken in immediate succession. With HDO the valve adjusts in real time over the entire measurement cycle to achieve linear deflation rate over the pressure range (0 – 300 mm Hg).

1.5.3b Blood pressure data acquisition

A minimum of three, maximum of five consecutive blood pressure readings were recorded for analysis.

1.5.3c Blood pressure data analysis

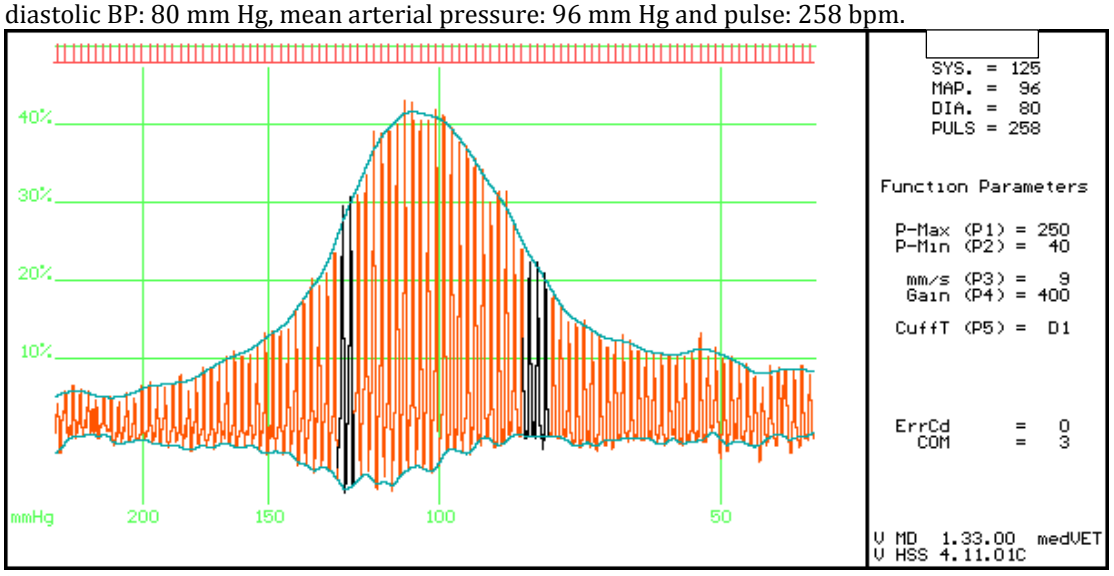
The system's internal microprocessor calculates blood pressure. Systolic, diastolic, mean arterial pressure and pulse rate are recorded by the ECG technician at the time of acquisition from the digitized display on the blood pressure machine and from the real time trace, each individual wave viewed for accurate marker placement and determination of SYS and DIA (Figures 1.5.2a - d).

[1] The tail was chosen because the coccygeal artery lies on the vertebrate and is therefore close to the skin with little muscle or fat, affording good uniformity of arterial pressure when compressed with the cuff.

1.5.3d Trace quality

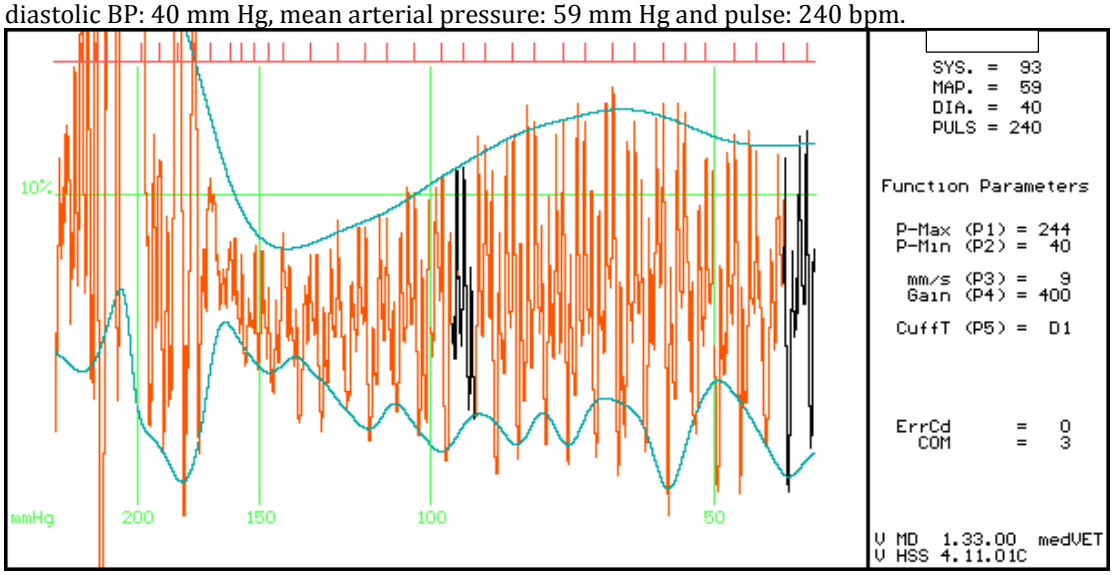
Movement artefacts and muscle tension create problems when measuring blood pressure, as with recording ECG (Section 1.3.2d). Figures 1.5.2b - d show common artefacts experienced during recording HDO blood pressure from macaques.

Figure 1.5.2a Real time HDO blood pressure trace recorded from a conscious, tube restrained cynomolgus macaque. SYS and DIA are marked in blue. Systolic BP: 125 mm Hg, diastolic BP: 80 mm Hg, mean arterial pressure: 96 mm Hg and pulse: 258 bpm.



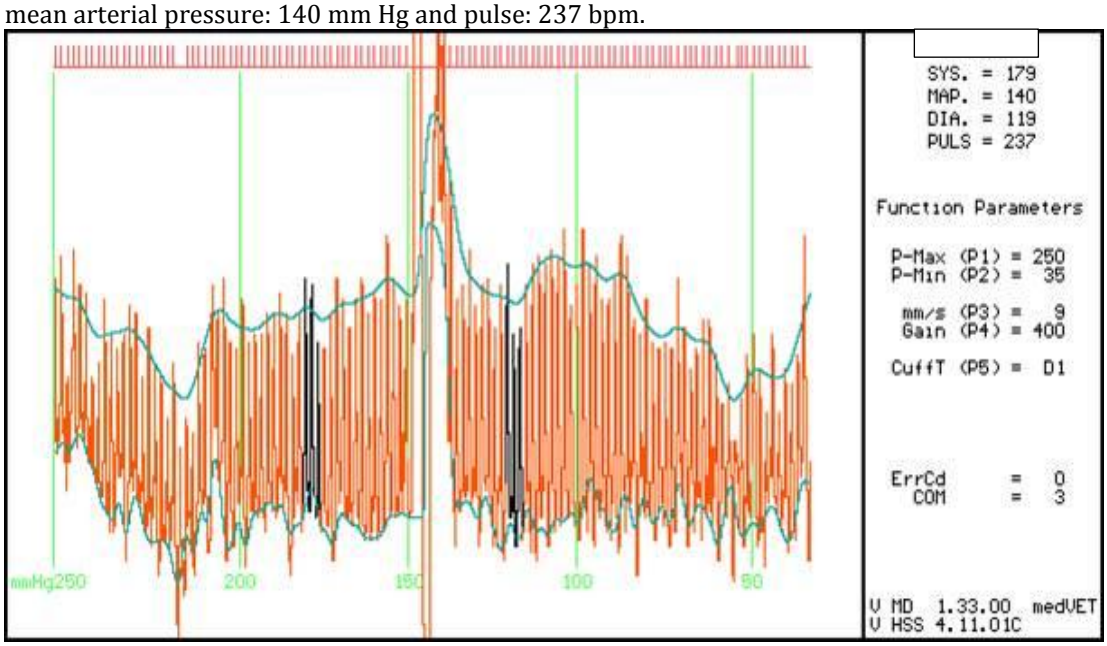
'Clean' trace with no movement artefacts, blue marks indicate the processor has determined systolic and diastolic blood pressure from the correct portion of the trace.

Figure 1.5.2b Real time HDO blood pressure trace recorded from a conscious, tube restrained cynomolgus macaque. SYS and DIA are marked in blue. Systolic BP: 93 mm Hg, diastolic BP: 40 mm Hg, mean arterial pressure: 59 mm Hg and pulse: 240 bpm.



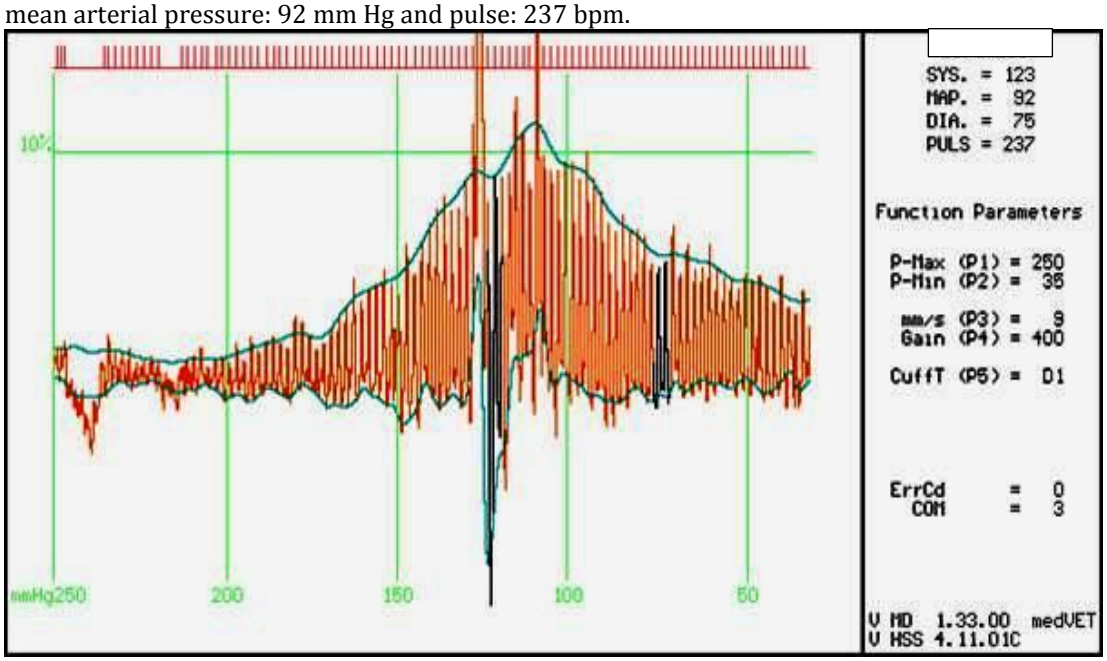
Poor quality trace, the blood pressure readings are inaccurate. The recording will need to be repeated prolonging the procedure.

Figure 1.5.2c Real time HDO blood pressure trace recorded from a conscious, tube restrained cynomolgus macaque with movement artefacts due to sustained animal movement. SYS and DIA are marked in blue. Systolic BP: 179 mm Hg, diastolic BP: 119 mm Hg, mean arterial pressure: 140 mm Hg and pulse: 237 bpm.



'Noisy' trace owing to movement artefacts. The microprocessor is not able to take an accurate reading. The recording will need to be repeated prolonging the procedure.

Figure 1.5.2d Real time HDO blood pressure trace recorded from a conscious, tube restrained cynomolgus macaque with movement artefacts due to macaque vocalisation (alarm call). SYS and DIA are marked in blue. Systolic BP: 123 mm Hg, diastolic BP: 75 mm Hg, mean arterial pressure: 92 mm Hg and pulse: 237 bpm.



Macaque vocalised as the microprocessor records systolic blood pressure (first blue mark). The recording will need to be repeated prolonging the procedure. From Bodey (2010).