THE IMPACT OF FLUID RESTRICTION PROTOCOLS ON RHESUS MACAQUES (MACACA MULATTA) AND REFINEMENTS TO THEIR USE

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Abstract

Animal models are an integral component of modern science. Non-human primates (NHPs) are effective models for many human diseases and conditions due to their close phylogenetic relationship. In particular, their specific cortical organisation and neural specialisations makes them invaluable for neuroscience research, both basic and applied. The advanced cognitive abilities of NHPs and their fine motor dexterity means that they can be trained to perform complex tasks in the laboratory whilst cortical activity is measured. Many of these tasks require hundreds or thousands of iterations in order to achieve statistical power to adequately test hypotheses, and consequently, the monkeys need to be sufficiently motivated to perform. One way in which researchers motivate their monkeys is through the use of fluid restriction protocols. By limiting the free intake of fluids, fluid rewards can be used as a primary motivator for the monkeys to continue to perform the tasks. These restriction protocols, although widely used, remain controversial due to their potential negative impacts on animal welfare. The aim of my thesis was to explore the impacts of fluid restriction protocols on rhesus macaque (*Macaca mulatta*) behaviour and physiology and to investigate possible refinements to their use.

My experiments found no evidence of negative physiological impacts of fluid restriction protocols and only limited impact on behaviours, alleviating some of the concerns surrounding these procedures. I also assessed the use of preferred fluids and social stimuli (photographs and video clips of conspecifics) as rewards. Mixed results were gained when assessing fluid preferences and again when implementing the preferences into laboratory tasks. Preferences for social stimuli were established for all animals tested, but these did not translate into motivating rewards on a trial-by-trial basis.

These studies have tackled important scientific and ethical issues surrounding the use of rhesus macaques in behavioural neuroscience. The outcomes are discussed in a wider context and the potential applications to laboratory practice are evaluated.

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Contents

Abbreviations

%	Percentage
µmol	Micromoles
ADH	Antidiuretic Hormone
ANOVA	Analysis of variance
CANTAB	CAmbridge Neuropsychological Test Automated Batteries
cd/m^2	Candela per square metre
CfM	Centre for macaques
CIE	Commission internationale de l'éclairage (international commission on illumination)
CNS	Central nervous system
Cr	Creatinine
DALY	Disability-adjusted life years
df	Degrees of freedom
DPZ	Deutsches Primatenzentrum (German Primate Centre)
dva	Degrees of visual angle
EU	European union
FAI	Free Access Intake
FAWC	Farm Animal Welfare Council
FDR	False discovery rate
h	Hours
HCT	Haematocrit
HD	Huntington's disease
hz	Hertz
IM	Intra-muscular
Kg	Kilogram
L	Litre
LMM	Linear mixed model
m	Metre
mg	Milligram
min	Minutes
ml	Millilitres

mmol	Millimoles
mOsmol	Milliosmoles
MRI	Magnetic Resonance Imaging
ms	Millisecond
MS	Multiple sclerosis
N	Sample size
Na	Sodium
NACWO	Named animal care and welfare officer
NC3Rs	National Centre for the 3 Rs
NHP	Non-human primate
NS	Non-significant
Osm	Osmolality
PETA	People for the Ethical Treatment of Animals
RGB	Red, Green, Blue
s	Second
SEM	Standard error of the mean
SG	Specific gravity
SM	Service mark
Std	Standard
v	Version
VR	Variable ratio
vs	Versus
WHO	World health organisation
YLD	Years lost to disability

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Chapter 1: General Introduction to the Use of Non-Human Primates in Research

1.1 Non-Human Primates as Model Species in Research

Animal models are a crucial, and currently irreplaceable, facet of science. Although extensive efforts are being made to reduce the numbers of animals used in research, this process is likely to take many years. In addition, a limited number of *in vitro* and *in silico* solutions exist, and for some areas of study, alternatives are not easy to develop, leaving *in vivo* research as an indispensable tool for our understanding of human and animal disease and development. Willner (1984) proposed that animals must satisfy three main criteria to be effective as models and although these criteria were created with psychological disorders in mind, they are often applied more widely. Firstly, a model must have face validity; the ability to exhibit the symptoms and behavioural signs of the disease or condition being modelled. Secondly, it must have etiological or construct validity; meaning that the underlying cause of a condition is similar in the model to in the human. Finally, predictive validity is required to demonstrate that treatments or interventions known to cause effective reversal or alleviation in humans are mirrored in the model. For many diseases and conditions, genetically modified rodents, with specific genes knocked out or silenced, can provide useful models for translation into humans (Proetzel and Wiles, 2010). However, in certain circumstances, the phylogenetic distance between humans and rodents may be too great for the animal to effectively simulate the human condition.

One group of animals which, in many situations, fulfil the criteria of effective models is the non-human primates (NHPs). NHPs comprise a wide range of species, broadly categorised into Prosimians (including the lemurs, lorises and tarsiers), New World monkeys from South and Central America, Old World monkeys from Africa and Southern Asia, and apes (including our nearest relatives, chimpanzees (*Pan* spp.) and gorillas (*Gorilla* spp.)). Of these, only species of New and Old world monkeys are used for scientific research in the United Kingdom (UK), with macaques (*Macaca* spp.) and common marmosets (*Callithrix jacchus)* being the most commonly used models (Weatherall et al., 2006). Prosimians have not been used since 1991 in the UK and great

apes since 1986, with a complete UK ban on great ape research in 1997 (Weatherall et al., 2006).

NHPs are of particular importance to science as their phylogenetic relatedness to humans improves their efficacy as animal models in a range of circumstances (VandeBerg and Williams-Blangero, 1997). For example, the baboon (*Papio* spp.) provides an excellent model for osteoporosis, as characteristics of the disease spontaneously develop in older females, as they would in humans, satisfying the criterion of face validity (reviewed by VandeBerg and Williams-Blangero, 1997). Due to their wide behavioural repertoire, NHPs also provide excellent opportunities for the study of certain manipulations or treatments at the behavioural level (etiological validity), with rhesus macaques (*Macaca mulatta*) and cynomologous macaques (*Macaca fascicularis*) being the most used NHP species for this purpose (Carlsson et al., 2004). Finally, a range of NHP species and their related behaviours have been used to tackle important scientific questions such as addiction, depression, drug treatments, adoption, ageing and abnormal development, proving their predictive validity as a model (as reviewed by Hau and Schapiro, 2006).

1.2 Non-Human Primate Usage

NHP research occurs worldwide and although specific figures are not available from all countries, the following statistics give an impression of the numbers of NHPs used, the most common species employed and the fields to which NHPs contribute the most.

Globally, in 2001, approximately 100,000 to 200,000 NHPs were estimated to have been used in scientific study, following an extensive retrospective literature review by Carlsson et al. (2004). The majority of these studies were microbiological (26%) and neuroscience studies (19%), with 37% of all studies using either rhesus macaques or vervet monkeys (*Chlorocebus aethiops*) (Carlsson et al., 2004). However, in microbiology, the use of *C. aethiops* usually refers to the study of "primate biological material" and often involves the use of cell lines from culture or museum specimen samples rather than a conscious animal (Carlsson et al., 2004). This effectively leaves neuroscience as the largest field, worldwide, which uses awake and behaving NHPs for data collection.

In the USA, approximately 62,000 NHPs were used in 2015 (United States Department of Agriculture - Animal and Health Inspection Services, 2016) and a review of 26 academic and private research facilities by Lankau et al. (2014) reported that 89% of the facilities conducted pharmaceutical research and studies relating to neuroscience, neurology or neuromuscular disease. Facilities also reported high levels of investigation into vaccine development and testing (62% of facilities), pharmaceutical preclinical safety research (50% of facilities), and immunology or autoimmune disease research (42% of facilities). In line with global species use, 81% of the facilities used rhesus macaques and 73% used cynomologous macaques. These figures highlight the particular importance of macaque species in research and the wide variety of contexts in which they can be utilised.

Approximately 2.08 million experimental procedures were carried out on animals in the UK in 2015 (excluding breeding and creating), however only 3612 (0.09%) of these used NHPs (marmosets and macaques) (UK Home Office, 2016). Similarly, of 2.01 million individual animals used for the first time, 1.26 million were mice (74.6%) but only 2234 were NHPs (0.05%). These figures include all types of scientific procedure and when further subdivided by study type, only 94 NHPs were used for basic science of the nervous system and 8 for the applied study of human mental disorders. The lower numbers of NHPs used compared with other species is due, in part, to strict European Union (EU) regulations which state that research cannot be carried out with primates if the equivalent results can be gained in a lower species (European Union, 2010). Additionally, conducting research with NHPs in the UK imposes not only the required regulation compliance, but also high financial costs. A rhesus macaque costs approximately £20,000 + VAT to buy and around £300 per week for housing costs (Personal communication with Named Animal Care and Welfare Officer (NACWO)), compared with $US 1000 dollars per animal in China (approximately £700) and significantly lower housing costs of $5 per day (Cyranoski, 2006). In comparison, a mouse in the UK costs from approximately £2–£200 per individual (dependent on strain and rearing; prices from Charles Rivers, Research Models and Services, UK) and colony costs are roughly £1.50/mouse/week. Taken together, these factors help to explain why the numbers of NHPs used in research are lower than the numbers of other model

species. Despite this, NHPs remain an indispensable asset to research, continuing to contribute to numerous scientific findings of global importance.

The aforementioned phylogenetic similarities between humans and NHPs have aided in the success of many recent medical discoveries. NHPs have contributed extensively to numerous branches of science, helping to develop life-saving vaccines for diseases such as Hepatitis B (Prince and Brotman, 2001) and Polio (Bayley, 1956) and facilitating the development of safe organ transplantation (Knechtle, 2000; Haanstra and Jonker, 2008). More recently, NHPs have contributed towards research to combat the Ebola (e.g. Jones et al., 2005) and Zika crises (e.g. Osuna et al., 2016). However, this thesis is specifically concerned with the use of NHPs in neuroscience research, for which they are a widely used and a highly effective model, particularly for understanding cognitive functions, brain disease, and to aid potential therapies in humans (Roelfsema and Treue, 2014).

1.3 The Impact of Brain Disorders

It can be argued that the importance of neuroscience research, both basic and applied, is more important now than ever, due to the increasing economic and social impacts of brain disorders. In a systematic review, Gustavsson et al. (2011) calculated that common brain disorders (including mood disorders, dementia, psychotic disorders and anxiety disorders) cost the EU €798 billion in 2010. Not only is this a financial burden, but with an estimated 38.2% of the European population suffering from a form of brain disorder at some point during their lifetime (Wittchen et al., 2011), these illnesses are also a source of social and emotional difficulties for the millions of people that they afflict. These problems are also consistent across the EU; with the exceptions of mental retardation and substance abuse disorders, mental illnesses and their associated economic impacts do not differ across the member states, emphasising their widespread prevalence (Wittchen et al., 2011).

On a global scale, the World Health Organisation (WHO) estimated that in 2001, approximately 450 million people were known to suffer from neurological and brain disorders (World Health Organisation, 2001). In 2010 mental disorders and substance abuse resulted in 7.4% of all disability-adjusted life years (DALYs - a measure of disease burden, the sum of the years of lost life due to premature mortality; and years lost to

disability (YLD)) (Whiteford et al., 2013) and in 2013, major depression was revealed to be the second greatest cause of YLD out of all illnesses and conditions (Vos et al., 2015). Unfortunately, the dominance of these illnesses is proliferating. From 1990 to 2013 the occurrence of neurological disorders increased by 59.6% and mental and substance abuse disorders by 45%, largely a product of ageing and population growth (Whiteford et al., 2013). Reflecting this, Alzheimer's disease and Parkinson's disease were calculated to affect 53 million and 5.9 million people, respectively, in 2013 (Vos et al., 2015). The economic impacts mirror the social burdens, as neurological disorders cost $2.5 trillion globally in 2010, and a report from Harvard School of Public Health and the World Economic Forum calculate this to increase to $6 trillion by 2030 (Bloom et al., 2011). These figures highlight that the scale of the problem is vast, and that extensive research into brain disorders is required to help to understand and effectively treat these conditions.

1.4 Non-Human Primates in Neuroscience

In order for neuroscience studies to be more effectively translatable to humans, it is beneficial for the animal models to possess certain characteristics; NHPs exhibit many of these vital characteristics, the majority of which are lacking in rodent models. For example, like humans, NHPs have forward-facing eyes, a fovea, and similar visual cortical organization (Zeki and Shipp, 1988). NHPs also exhibit smooth pursuit eye movements, necessary for some cognitive function studies (Kettner et al., 2008) and possess a prefrontal cortex homologous to humans, the presence of which is debated in rats (Preuss, 1995). Certain neuropharmacological specialisations are also comparable between macaques and humans, but differ profoundly from rodents (Disney et al., 2006). Recent research has also uncovered that primate brains are unique in their neuronal density structure; as the number of neurons increases, the density remains the same (Herculano-Houzel et al., 2014).

1.4.1 The Contribution of NHPs to Neuroscience

The importance of NHPs in contributing to neurological research has been well-established for a number of years. Notably, Hubel and Wiesel conducted ground-breaking work into visual processing in the spider monkey and the macaque which led to their Nobel Prize for Physiology and Medicine in 1981 (Nobel Foundation). Since then,

NHPs have contributed profoundly to advancements and achievements in neuroscience (see Capitanio and Emborg, 2008; Kettner et al., 2008; Camus et al., 2015 for reviews). Recently, Roelfsema and Treue (2014) stressed the importance of the need for NHPs in basic neuroscience research; this uncovers the fundamentals of mechanisms or pathways in the brain which can then be studied in a more applied manner to focus on specific disorders or treatments. By conducting basic neuroscience in NHPs, a number of significant discoveries have been made, including increased knowledge of intra-cortical connectivity, as well as uncovering mechanisms of object recognition and decision making (Roelfsema and Treue, 2014).

In addition to basic neuroscience, NHPs are also central to the study of particular medical conditions. For example, using NHPs in autism research provides a bridge between well-established mouse models and studies of human patients, allowing for the assessment of behavioural outcomes of specific manipulations and interventions (Watson and Platt, 2012). The study of multiple sclerosis (MS) has also benefitted from an NHP model of the condition. MS is a disease affecting the brain and spinal cord, where invasive studies in human patients cannot be undertaken. Although rodent models do exist, it is through the development of a specific marmoset model that more accurate investigation of the condition can be achieved ('T Hart et al., 2004). NHPs also provide excellent opportunities for the study of ischaemic stroke in humans, not only due to their similar brain structures, but due, also, to the parallels in vasculature (Fukuda and del Zoppo, 2003). Finally, in the last decade, NHP neuroscience research has been further advanced by the development of transgenic models. For example, Yang et al. (2008) established a transgenic macaque model of Huntington's disease (HD) and successfully induced the physiological features of HD, as well as the clinical symptoms, providing a platform for more comprehensive study of the condition. Although many more examples exist, it is clear from this brief synopsis that the use of NHPs in neuroscience has brought a great deal of benefit and understanding to the scientific community, which can be translated into benefits for society.

1.4.2 Task Performance in Neuroscience

In addition to the similarities in brain structure, a further key advantage of using NHPs in neuroscience is that they possess advanced cognitive and motor abilities conducive to training them to perform a number of complex tasks in the laboratory. This

allows tasks originally designed for human participants to be successfully adapted for NHPs, facilitating more in-depth analysis of the development of certain conditions or pathways. For example, Diamond and Goldman-Rakic (1989) modified Piaget's A-not-B task (a task used to investigate stage four object permanence) for use with macaques. This allowed comparisons to be made with data from human infants, and helped to identify the probable brain area underlying the development of A-not-B task performance. Furthermore, studies have used tests based upon the CANTAB battery (CAmbridge Neuropsychological Test Automated Batteries; CeNeS, Cambridge, UK), designed for human use, to test neuropsychological functions in NHPs (e.g. Weed et al., 1999). Multiple aspects of cognition can be assessed by using this battery of tasks, such as memory, decision-making, and attention. By employing these tests in conjunction with careful manipulation of the NHP central nervous system (CNS), specific areas or pathways associated with certain disorders can be identified in a way that is not feasible in humans.

To understand brain processes whilst a specific task is being performed, many neuroscience studies implement electrophysiology or neural imaging. Electrophysiology involves recording activity from single or multiple neurons using electrodes placed into the relevant area of the cortex. Imaging techniques, such as magnetic resonance imaging (MRI), require a monkey to engage in a task whilst situated in a brain scanner. The nature of these methods normally necessitates an animal to have its head fixed in a set position in order to minimise movement whilst recordings are taking place. In these types of studies, good quality data collection and adequate statistical power requires the need for a high number of consecutively performed trials from the monkey. This is especially true in electrophysiology, where a single cell recording session cannot be replicated on another day. For this reason, a training, imaging or recording session can last for several hours (Kettner et al., 2008). Low numbers of daily trials can render recordings unusable, wasting time and money, and keeping an animal's head fixed in a primate chair for little reason. Therefore, laboratories must use reliable techniques to motivate their monkeys in order to acquire data from a sufficient number of trials per day. One way of successfully achieving high motivation is through the implementation of fluid or food restriction protocols.

1.5 Restriction Protocols

1.5.1 The Use of Restriction Protocols

Restriction protocols involve limiting the amount of fluid or food that an individual animal consumes daily. These protocols should not be confused with food or fluid deprivation, which completely deny access to food or fluid for a set period of time. Restriction protocols are usually implemented in one of two ways: 1) food or fluid gained through work is accessible for a pre-defined time per day (e.g. 3 h access); or 2) animals are allowed to work for as much food or fluid as they desire. This thesis is focussed on fluid restriction relating to option 2), specifically. When animals are subject to fluid restriction, correct trials performed by the animal are rewarded with a droplet of liquid, allowing the researcher to use motivation to drink as a primary motivator to work (Toth and Gardiner, 2000; Rowland, 2007). For more complex tasks, or those requiring high levels of repetition, stricter restriction protocols may have to be used in order to maintain engagement in the task and promote effective learning and a reliable performance of trials (Toth and Gardiner, 2000). For an example from the rodent literature, Hughes et al. (1994) conducted a study in which rats subject to differing levels of fluid restriction had to press a lever to gain access to water. Those rats on a more restrictive regime (21 h restriction/day) learnt the task well. However, rats restricted less harshly (7 h or 14 h/day) failed to perform the task. In this example, a high level of restriction was required to gain a reliable response from the rats, in what was only a mildly challenging task. It would be expected that a more complex task would require an even higher level of restriction.

For certain behavioural neuroscience studies using NHPs, it may be more practical and effective to use fluid, rather than food, rewards. Although there are instances in which food rewards can be successfully implemented, this is not always possible when performing studies requiring the monkey's head to be fixed in a set position. The manual presentation of food rewards, and the subsequent chewing of food items can disrupt both stimulus presentation and data collection. For these reasons, along with the fact that fluid restriction is easy to measure and to control for both researchers and husbandry staff, fluid rewards are widely used.

1.5.2 Welfare Measures Concerning Fluid Restriction

Despite the widespread use of fluid restriction protocols, and the justification for their implementation, their use, especially with NHPs, has been an issue of increasing contention for over 20 years (Orlans, 1991; Desimone et al., 1992; Evans, 1993). The major concerns voiced include potential dehydration (Rowland, 2007), weight loss (Prescott et al., 2010), and pain or distress (Willems, 2009). Potential dehydration reflects a concern directly resulting from fluid restriction; that limiting water access will cause physiological harm to the animal. Weight loss as a result of fluid restriction normally refers to a voluntary decline of food consumption as a consequence of a decreased fluid intake (described in more detail in Chapter 3, sections 3.1 and 3.5.2). Willems (2009) claims that the types of pain or distress inflicted upon an animal via fluid restriction may include: agitation, altered aggression and lethargy. Other concerns include the potential for the animals to binge eat and drink when given access to larger volumes of food and water, with the potential to cause bloat and discomfort (Prescott et al., 2010). All of these concerns relate directly to infringements of The Five Freedoms, a framework originally developed for farm animals by the Farm Animal Welfare Council (FAWC), which is now widely applied to captive animals (Farm Animal Welfare Council, 1992). The Five Freedoms comprise of the following: 1) Freedom from hunger and thirst; 2) Freedom from discomfort; 3) Freedom from pain, injury or disease; 4) Freedom to express normal behaviour; and, 5) Freedom from fear and distress. However, despite the potential violation of the five freedoms, the long-standing controversies, and the possible animal welfare issues associated with fluid restriction, there exists a paucity in data investigating the impacts of the protocols.

Only by addressing the scarcity in data and understanding the impact of fluid restriction on NHPs, can we estimate how much stress, physiological or psychological, these protocols may cause. It is of great importance to understand the implications of any technique used in animal research so that refinements to the protocols may be attempted, in order to minimise any potential welfare issues. Refinement constitutes one of the 3Rs of animal research; a concept introduced by Russell and Burch in their seminal paper in the 1950s (Russell and Burch, 1959). The 3Rs consist of the replacement, reduction and the refinement of the use of animals in scientific research and they have been adopted as key aims for the progression and development of *in vivo*

research. Assessing and refining fluid restriction techniques used in behavioural neuroscience is particularly important for two main reasons. Firstly, if fluid restrictions were to cause physiological or behavioural distress or harm to the animal, welfare would be compromised, creating an ethical concern with the procedure. Secondly, the scientific validity of the research for which the animals are primarily being used could be impacted upon if fluid restriction protocols result in increased levels of stress. For example, animals that exhibit stereotypic behaviours (explained in more detail in Chapter 3, sections 3.1 and 3.5.3), a marker generally used as an indicator of poor welfare, may not produce valid, reliable or replicable results in scientific study (as reviewed by Garner, 2005).

The importance of investigating the impact of procedures on laboratory NHP welfare is not only important for the NHPs and the scientific community, but for the public's understanding of primate research. Primate use is an emotive subject and in the last two decades many NHP researchers have found themselves subject to increasing pressure from animal rights activists and the general public, campaigning for them to justify or end their work (e.g. Cyranoski, 2006; Abbott, 2014). In addition to targeting scientists, protests of animal rights groups such as PETA (People for the Ethical Treatment of Animals) have also focused their efforts on the source of the monkeys, causing airlines to cease the transportation of NHPs and making the supply to research facilities increasingly difficult (Wadman, 2012). The somewhat turbulent public perception of primate studies has resulted in many reviews and commentaries in defence of NHP use in neuroscience research. For example, Camus et al. (2015) defend the use of NHP models for cognitive neuroscience, citing the major advancements made in methodologies and technologies for neuroscience research, as well as the need for NHPs for the study of debilitating conditions such as Alzheimer's and Parkinson's disease. However, by publishing in scientific journals, the authors fail to reach many of those who oppose the research. Only by clarifying the use of NHPs in scientific study and by making every effort to assess and, where necessary, improve their welfare, can we begin to create a useful dialogue between researchers and those who oppose their work. It is for these reasons, both scientific and ethical, that this thesis will investigate the use of fluid restriction protocols to motivate rhesus macaques in behavioural neuroscience.

1.6 Aims

There are important gaps in knowledge surrounding fluid restriction protocols and there exists the need for clear and applicable research regarding the welfare of laboratory NHPs and the protocols imposed on them. Due to the widespread use of fluid restriction protocols, the controversy of their practise and their potential impact on animal welfare, it is the aim of this thesis to expand the current understanding of fluid restriction and to investigate potential refinements to the technique.

Specifically, the three main areas that will be addressed in this thesis are:

1. To explore the impact of fluid restriction protocols on the behaviour, physiology and welfare of rhesus macaques used in behavioural neuroscience and to assess the scientific output of monkeys undergoing different fluid restriction protocols.

2. To test for fluid preferences in the macaques and investigate the potential motivational value of preferred fluid rewards and to assess whether these can be used to refine fluid restriction protocols.

3. To assess whether non-nutritive rewards, in the form of social stimuli (images and video clips of conspecifics), can be utilised alongside, or instead of, current fluid restriction protocols.

These studies were conducted in a laboratory where rhesus macaques were used to understand higher cognitive functions and the neuropharmacology of cognitive functions in the context of visual processing. Consequently, whilst the experiments described in the following chapters were designed with the principle aims of the thesis in mind, studies were incorporated into existing neuroscience studies, where possible, in order to reduce the use of the animals undergoing experimentation.

Chapter 2: General Methods

2.1 Ethical Statement

All experimental animal procedures complied with European Union Directive 2010 (2010 63 EU), the National Institutes of Health (*Guidelines for the Care and Use of Laboratory Animals*), the Society for Neurosciences Policies on the Use of Animals and Humans in Neuroscience Research, and the Animals (Scientific Procedures) Act 1986. All reporting abides by the ARRIVE guidelines and work was carried out under a UK Home Office approved and regulated project license.

2.2 Animals

All animals used in this thesis were rhesus macaques, aged between 4 and 9 years old and weighing between approximately 4 and 15 Kg. Animals were used in behavioural neuroscience studies, and were all experienced in the experimental set-ups and behavioural tasks. The animals were pair-housed and the cages in the facility located such that the individuals could obtain visual and auditory contact with other monkeys. The monkeys were provided with toys on a rotated basis as environmental enrichment, and dry food mix was placed in the floor covering to allow them forage. This has been shown to be stimulating and rewarding (Chamove and Anderson, 1989) and has been recommended by primate welfare guidelines (NC3Rs, 2006). The home cages were one of two sizes: 2.1 x 3.0 x 2.4 m or 2.3 x 2.45 x 2.4 m and the facility was lit on a 12 h light/dark cycle with additional light from ceiling windows. The temperature and humidity were approximately 20 °C and 24%, respectively.

Animals in the facility undergo daily checks by a technician or veterinarian even in the absence of any health or welfare concerns. Fur condition, faeces, eyes, food intake and activity levels are all visually assessed. Any time there is a health or welfare concern, or if the animal is in a post-operative period, technicians and the veterinarian check the animal several times per day. In these circumstances, wound healing is also assessed. In addition to daily checks, all primates are blood sampled annually to test for tuberculosis. Blood samples are also assessed for levels of the following: white blood cells, red blood cells, potassium, calcium, urea, cholesterol and proteins. All animals were tested for

viral and bacterial zoonoses, including *Salmonella* spp., *Campylobacter* spp. and *Shigella* spp., at their breeding facility.

Throughout this thesis the monkeys in each chapter are referred to as Monkey 1, Monkey 2 etc. Each chapter treats the naming of monkeys separately, and thus, Monkey 1 in Chapter 3 is not necessarily the same individual as Monkey 1 in Chapter 4.

2.3 Fluid Restriction Protocols

Fluid restriction protocols need to be tailored to each individual animal, to ensure maximum motivation with minimum restriction severity. For each animal, the volume of water consumed under free access conditions (free access intake [FAI]) was determined over a period of at least five (not necessarily consecutive) days. Following this, starting at a minimum of 70% FAI, the animal's performance in the experimental setup was determined over at least 3 days. The minimum was then decreased as necessary (in steps of 10-15% of FAI) until the animal was sufficiently motivated to work for fluid rewards in order to obtain scientifically useful data (approximately 1000-1200 correct trials in a daily session). After each reduction, the animal's work rate was assessed for at least 3 working days to determine current levels of motivation and performance. Only if the current minimum was insufficient to achieve the required number of daily trials, were further decreases implemented. In cases where motivation required a drop to 30% or below of the free access intake, the named veterinary surgeon was contacted to assess the impact that this reduction would have on the animal's welfare.

Animals worked 5 days per week (Monday – Friday) throughout the experiments. Within a daily experimental session, the monkey was allowed to work for as much fluid as he wanted, but in situations where the minimum daily allowance was not earned during the task, the monkey was supplemented (to its established minimum) with water in the laboratory after the session had finished. Therefore, monkeys received at least their minimum fluid allowance every working day. The monkeys received their minimum allowance amount either in the laboratory (Monday to Thursday) or in the home cage (Sunday). On Friday evenings and Saturdays, they were given free access to water in the home cage. This changed only for the experiments in Chapter 2, which

were designed specifically to investigate different fluid restriction protocols. These additional protocols are described in detail in Chapter 3.

2.4 Apparatus

All testing was carried out in the laboratory whilst the monkeys were seated in a custom made primate chair with their heads fixed by a post set in dental acrylic. Headpost surgery was carried out with 1 – 3% sevoflurane general anaesthesia and under aseptic conditions, previously described by Thiele et al. (2006). Testing was carried out in a dimly lit room with ambient light level at ~ 3-5 cd/m^2**,** to ensure adequate contrast detection during tasks. The stimuli were presented on a Iiyama HM204DTA computer monitor, with an 85 Hz refresh rate and 1280 x 1024 pixel resolution. Stimulus presentation, reward delivery and experimental timing were controlled on IBM-compatible personal computers, using the Cortex programme (DOS-Version 5.95; IMH, http://dally.nimh.nih.gov/). Monkeys were weighed daily prior to each experimental session (Mon-Fri), and then transferred between the housing unit and the laboratory using a custom-made trolley, onto which the primate chair was fitted.

Chapter 3: The Impact of Fluid Restriction Protocols on the Physiology, Behaviour and Scientific Output of Rhesus Macaques

3.1 Introduction

As discussed in Chapter 1 (section 1.5), fluid restriction protocols are a widely used motivational technique used in primate behavioural neuroscience, although their impact on animal welfare is poorly understood and contentious. In an NC3Rs Working Group report of 2010, Prescott et al. (2010) identified gaps in knowledge concerning the use of fluid restriction with NHPs, highlighting the paucity of data regarding how these protocols might impact on NHP welfare. There are several concerns surrounding fluid restriction protocols, including dehydration, weight changes and impacts on behaviours in the home cage.

The first concern to address is the potential to cause dehydration(Prescott et al., 2010). Measures of dehydration and mechanisms of thirst are well-studied in the rhesus macaque (Wood et al., 1982). The physiological mechanism of thirst in the macaque is caused mostly by cellular dehydration, with reduction in plasma volume contributing a smaller effect (Wood et al., 1982) but the two processes are linked, so although cellular dehydration cannot be measured directly in task-performing laboratory animals, clinically validated proxies are available. For example, the concentrations of ions in the bloodstream are highly correlated with cellular dehydration but are much simpler to measure (Wood et al., 1977). The levels of sodium, haematocrit, urea and creatinine in the blood increase as fluid intake is decreased, due to a lowered volume of water in the bloodstream. To then maintain homeostasis of the blood, compensatory changes in urine concentration are expected. This occurs when decreases in fluid intake are detected by osmoreceptors in the anterior hypothalamus, which in turn cause the posterior pituitary to secrete antidiuretic hormone (ADH). ADH causes kidney cells to reabsorb water in to the blood, resulting in more concentrated urine. If fluid restriction protocols impact upon macaque physiology and result in adaptive responses to conserve fluids, urine osmolality, creatinine and specific gravity should increase.

Further concerns with fluid restriction protocols include possible loss of body mass (highlighted in Prescott et al., 2010). Fluid restriction could negatively impact on body condition, as consumption (especially of dry foods) may decrease if the monkeys experience increasing thirst. This voluntary decrease in food intake as a consequence of reduced fluid intake has been previously described in rodents and humans (Cizek and Nocenti, 1965; Collier and Levitsky, 1967; Engell, 1988).

In addition to the physiological and morphological impacts of fluid restriction, it is important to assess the effects on the monkeys' behaviour. Some behaviours change in predictable ways in relation to welfare. For example, as welfare declines, stereotypies increase (Lutz et al., 2003; Honess et al., 2004). Stereotypies are broadly defined as repetitive and seemingly functionless behaviours, such as pacing and rocking, and are often used as markers of compromised welfare (Mason, 2006). Inactivity and reduced energy are additional welfare indicators and increases in these behaviours can signal low mood; they are symptoms of depression in humans (Diagnostic and Statistical Manual for Mental Disorder, 2013). In addition, pharmacological trials have aided in identification of other behaviours associated with poor welfare. These include: displacement activities such as self-grooming, self-scratching, yawning, body shaking and eye rubbing, which all increase with drug-induced anxiety and which decrease with anxiolytic treatment (Schino et al., 1996; Palit et al., 1998). These so-called self-directed behaviours are also mirrored in the human stress phenotype (see Troisi (2002) for a review in NHPs and humans). Furthermore, a decrease in food consumption or foraging behaviours (as described above) may reflect a state of thirst. Many of the described behavioural measures have been previously used to assess substantial changes in an NHP's routine, such as air transportation and re-homing (Honess et al., 2004), effects of different social housing options (Schapiro et al., 1996; Baker et al., 2014) and the long term impacts of differential rearing conditions (Corcoran et al., 2012). In this study, I plan to test if the same measures can be used to detect behavioural changes in individuals undergoing common fluid restriction protocols.

There have been previous attempts to evaluate the use of fluid restriction on some aspects of animal welfare using physiological or behavioural measures. Yamada et al. (2010) found that increases in macaque blood osmolality caused by fluid restriction

quickly returned to normal levels during a rewarded behavioural task, and that osmolality remained mostly stable across a 5-day working week. More recently, Hage et al. (2014) failed to detect changes in home cage behaviour across a 12-day period of fluid restriction, although they were not able to compare these measures to behaviour during periods of free access to water. While both of these studies help to alleviate some concerns of fluid restriction protocols, it could be argued that they are too focused on one particular type of measure or too short-term to address concerns about longer-term impacts on welfare. There is a clear need to assess welfare over a longer period of time using a combination of measures to gain a more complete picture of the potential effects of fluid restriction. In order to shed light on this issue, physiological and behavioural measures sensitive enough to capture any changes in physiology or welfare must be used.

The experiment conducted in this chapter was designed to investigate the validity of the current concerns surrounding fluid restriction. The study implemented a controlled within-subject design in four macaques used in electrophysiological studies over a 16-week period. During this period, all four animals experienced two different fluid restriction protocols which are commonly used in primate research (Prescott et al., 2010). The physiological and behavioural outcomes of these fluid restrictions were compared with baseline data taken when the monkeys had free access to water. In addition, some physiological measures were compared to two 'control' groups. The first was a non-restricted, age- and sex-matched control group at the Centre for Macaques (CfM) UK breeding facility to ensure that the macaques' data fell within a 'normal range'. The other was a sub-sample of monkeys at Newcastle University that were naïve to fluid restriction protocols to explore possible changes following long-term exposure to periods of fluid restriction. This chapter, therefore, describes a suite of physiological and behavioural measures to assess the impact of longer-term use of different fluid restriction protocols on rhesus macaque welfare, and how these protocols translate to performance in behavioural tasks and subsequent scientific data quality.

3.2 Materials and Methods

3.2.1 Fluid Restriction Protocols

Three different conditions were assessed: a control period of free access to water (hereafter "free access"), and both a 5-day and a 7-day fluid restriction protocol (hereafter "5-day protocol" and "7-day protocol"). The 5-day protocol consisted of 5 days of fluid restriction with free access to water on days 6 and 7. This is the standard protocol implemented in the laboratory of Prof. Thiele (and many other laboratories world-wide). The second protocol consisted of 7 days of fluid restriction, where animals had access to at least their individually established minimum every day (as described in Chapter 2, section 2.3), which they could exceed by means of their work-rate during experimental weekdays, but not on days 6 and 7. The 7-day protocol was tested to investigate potential costs and benefits in relation to welfare and scientific output. On a 5-day protocol, work rates following the two days of free access are normally too low to allow for electrophysiology recordings, leaving at least one day per week where the animal is fluid restricted and performs the cognitive task in the laboratory without usable data being collected. If a 7-day protocol was more effective at motivating animals to perform the task on a Monday, data collection could be quicker and periods of fluid restriction could be reduced. Given these potential benefits, it was important to be able to compare welfare measures between protocols, as well as to a control period of free-access.

On the 5-day protocol, subjects received their minimum fluid allowance either in the laboratory (Monday to Thursday) or in the home cage (Sunday). On Friday evenings and Saturdays, they were given free access to water in the home cage. On the 7-day protocol, the monkeys received their minimum fluid allowance every day (Monday to Friday in the laboratory and Saturday and Sunday in the home cage), but were never given free access to water. Protocols lasted for four weeks at a time and were repeated twice (total of 16 weeks of study, two x 4 weeks for each protocol). The protocols were given either in a 5-7-5-7 day order (two monkeys) or a 7-5-7-5 day order (two monkeys). The monkeys were sampled for blood and urine on the last Friday morning of each protocol (detailed below). After sampling (occurring every 4 weeks), they were given free access to water from Friday morning (after sampling) until Friday afternoon before

the next protocol began on Saturday. Free access was given for that period to aid recovery from ketamine sedation.

Animals worked 5 days per week (Monday – Friday) in the neurophysiological experiments. Within a daily experimental session, a monkey was allowed to work for as much fluid as he wanted, but in situations where the minimum daily allowance was not earned during the task, he was supplemented (to his established minimum) with water in the laboratory after the session had finished. Therefore, monkeys received at least their minimum fluid allowance every working day. For the 16 weeks of fluid restriction, monkeys were separated from their cage mates from Friday evening until Sunday afternoon. This was done to obtain accurate recordings of fluid intake for the monkey of interest and to ensure that the cage mate had adequate (unrestricted) access to water for that period.

Prior to the fluid restriction protocols, the monkeys experienced a control period of 12 days during which they had free access to water, and behavioural and physiological measures were taken. A second control period of 12 days was implemented six months after the completion of the fluid restriction protocols, and physiological measures were taken again, and used with those from the first control period for analysis.

3.2.2 Tasks Performed by the Primates

For the duration of this study, each monkey was involved in ongoing neuroscience experiments, in which they were performing tasks in relation to visually presented stimuli to obtain fluid rewards. Monkeys 1 and 3 were rewarded with Ribena (Lucozade Ribena Suntory Ltd), Monkey 2 with water and Monkey 4 with diluted *coca-cola* (The Coca-Cola Company). Three subjects were engaged in covert top-down attentional tasks with individual trial times of 2000-4000 ms. The other monkey (Monkey 1) was performing a memory guided saccade task, with individual trials taking up to 5000 ms. Experiments were carried out in a dimly lit room. Performance in the laboratory was monitored via computer control; task performance, i.e. the number of correct trials performed by the monkey in their task, was recorded for each session. The criteria for determining when the monkey had stopped working (for example no consistent task engagement for > 15 min) differed slightly for each animal between experimenters, but

they remained consistent for individual monkeys over the course of the study. Experimenters were blind to which fluid restriction protocol their animal was currently subjected to and I provided the monkeys' water at weekends so that husbandry staff also remained blind.

3.2.3 Physiological Measures

Physiological measures of hydration state were collected at the end of the free-access periods (i.e. two data points per animal, one prior to implementing the fluid restriction protocols, the other 6 months after) and on the last day of each 4-week block of the 5-day and 7-day protocols (i.e. two data points/animal/protocol). To do this, animals were sedated with ketamine (10mg/Kg) intra muscular (IM) and blood was collected from the saphenous vein for haematological and biochemical analysis. During the sedation following the free access period, the bladder was located using ultrasound and urine was extracted via cystocentesis. During the fluid restriction protocols this was not possible due to the small size of the bladder and instead urine was collected from the cage on the morning of sedation, when possible. Urine was collected at least once per fluid restriction protocol for each monkey.

To compare results to a relevant baseline of non-restricted individuals, blood samples were also obtained from the Centre for Macaques (CfM), the UK rhesus macaque breeding facility. 14 male monkeys from 4-15 years old, weighing between 9-16 Kg were sedated as above and blood was collected from the femoral vein. The CfM monkeys received free access to water at all times and were group housed. Due to sampling and housing procedures, it was not possible to obtain urine samples from the monkeys at CfM.

Blood was also taken from two newly-restricted monkeys, i.e. monkeys previously naïve to fluid restriction, to ascertain whether impacts of fluid restriction were different when experienced for the first time. In these individuals, samples were taken (as described above) at 1 week from when the monkeys were first subject to fluid restriction and again at approximately 3 months and 6 months, dependent on scheduling this around the monkeys' progress in his cognitive task training, so as to not negatively impact on his development. These monkeys were 3 and 5 years old.

In order to assess any damage that fluid restriction may have caused to the kidneys, I obtained qualitative post mortem reports from two male rhesus macaques previously housed at Newcastle's facility and not included in this study. One monkey was of a similar age to those used in this study (8 years old) and the second was 16 years old; both had been fluid restricted intermittently on a 5-day protocol for 5 years and 11 years, respectively.

3.2.4 Weight Data

Animals were weighed on each weekday before being taken to the laboratory to evaluate weight change over the course of a working week as well as a longer-term assessment over the duration of a fluid restriction block (four weeks). The dataset was incomplete (due to occasional researcher absence or faults with the weighing scales) and the following number of weights were collected for each animal out of a possible 76 days (38 days per protocol, as animals were not taken to the laboratory, and thus not weighed, on physiological sampling days): Monkey 1: 65; Monkey 2: 75; Monkey 3: 67; Monkey 4: 74.

3.2.5 Behavioural Measures

In order to assess the potential psychological impact of different fluid restriction protocols, behavioural measures were collected while monkeys were in their home cages. Behaviour was recorded using cameras (Cube HD 1080, Y-cam) attached to the corridors of the primate housing facility, outside of each cage of interest. Data were collected three times per week: early week (Monday evenings and Tuesday mornings, to allow for husbandry procedures on Monday mornings), late week (Thursday morning and evening) and weekend (Saturday morning and evening). Using a range of days permitted assessment of changes in behaviour throughout the week. Morning recordings lasted from 07:00 – 09:00, and evening recordings from 17:00 – 18:40 (to coincide with lighting times). These times reduced the amount of personnel present in the primate facility, which, on its own could, affect animal behaviour.

An ethogram was designed to capture behaviours potentially associated with changes in welfare state (listed in Table 1) and behaviours were recorded using the Observer XT software (v 11, Noldus Information Technology). Behaviours were sampled

in one of two different ways. They were scored either every time they occurred in a video observation (hereafter called “continuous sampling”) or they were scored at a 30 second sample point (“scan sampling”). Continuous sampling was used for short or rare behaviours in order for them to be captured by the observation duration. Continuously sampled behaviours could be recorded either as ‘frequency’ data or as ‘duration’ data. Frequency data consisted of counts of behaviours, whereas duration data also included the length of time for which a behaviour was performed. A pilot set of behavioural observations (approximately 100 h of observations spread across all animals) was analysed to assess whether the full length of the recordings was needed to accurately capture potential behavioural changes induced by different protocols, or whether sampling the middle hour from the video collected was sufficient. Using paired *t* tests for each monkey (separately for both mornings and evenings of each fluid restriction protocol), no significant difference was found between analysing the middle hour (07:30 – 08:30, 17:20 – 18:20) and the full recording (all $t < 0.906$, $p > 0.378$; see Appendix A, Table 1). Therefore, observations and analyses were carried out using data from the middle hour only. In total, 410 h of video were observed and analysed with the following distribution across animals: Monkey 1: 108 h; Monkey 2: 112 h; Monkey 3: 90 h; and Monkey 4: 100 h. These numbers differ slightly due to some monkeys occasionally being brought back to the cage later than others which meant that they were not always present for the full video recording times. On rare occasions cameras also failed, resulting in lost footage. All videos were scored with the observer blind to the fluid restriction and all inter- and intra-rater reliability values were above 0.8 kappa score.

Table 1. Ethogram of behavioural measures of welfare for the rhesus macaque. Scan sampling occurred every 30 s and continuous sampling scored behaviours every time they were seen.

Category	Behaviours	Description	Sampling	Frequency /Duration
Inactive	Alert	Sitting/lying /standing stationary on any surface and looking at objects or individuals inside or outside of the cage.	Scan	Frequency
	Not alert	Sitting/lying/standing stationary on any surface, eyes may be open or closed, not looking at objects or individuals inside or outside of the cage.		Frequency
	Hunched	As for not alert, but sitting with head lower than the shoulders		Frequency
Foraging	Eating	Ingestion of items	Scan	Frequency
	Foraging	Searching for food or manipulation of food items or sources, without ingestion of food	Scan	Frequency
	Chewing	Chewing without any insertion of food into the mouth in the preceding 30 s	Scan	Frequency
Abnormal	Locomotor stereotypy	One or more completions of a repeated locomotor pattern, including any embedded behaviours	Scan	Frequency
	Other abnormal	Digit sucking, hair pulling, nail biting, rocking, head flicking, hand shake, any self-injurious behaviour	Continuous	Duration
	Self-groom	Stroking, picking, or otherwise manipulating own body surface	Scan	Frequency

Non-social behaviours	Self-scratching	Scratching the skin vigorously with nails	Continuous	Duration
	Yawn	Opening the mouth widely, teeth exposed, lips retracted without vocalisation	Continuous	Frequency
	Body shake	Dog-like body shake of whole body	Continuous	Frequency
	Eye rub	Rubbing the eye with a hand	Continuous	Duration
	Interact with physical environment – hands/feet	Swinging, pushing, manipulating any part of the cage or an enrichment with hands or feet without using mouth	Scan	Frequency
	Interact with physical environment - oral	Manipulating any part of the cage or an enrichment with mouth involved. Chewing/licking/biting any aspect of the cage or inanimate object in it.	Scan	Frequency
Social behaviours	Allogroom - donor	Stroking, picking, or otherwise manipulating a cage mate's body surface	Scan	Frequency
	Allogroom - recipient	Being groomed by cage mate, following above descriptors	Scan	Frequency
	Aggression to cage mate	Open mouth threat, chase, attack	Continuous	Duration
	Submissive to cage mate	Fear grimace, present, displacement of position in the cage	Continuous	Duration
	Aggression directed outside cage	Open mouth threat, attack or threat postures directed outside of the cage (e.g. at the glass)	Continuous	Duration
	Play with cage mate	Non-aggressive high intensity interaction (chase, wrestle, tumble) with cage mate	Scan	Frequency
	Mounting	Mounting cage mate	Continuous	Duration
	Being mounted	Being mounted by cage mate	Continuous	Duration

Locomotion	Agitated locomotion	Moving between locations, often rapidly, with a stiff un-relaxed gait	Scan	Frequency
	Relaxed locomotion	Moving between locations with a relaxed gait	Scan	Frequency
Other	Other	Any behaviour not listed above and noteworthy. Describe form.	Continuous	Duration

3.2.6 Water Bottle Approach and Consumption

In order to gauge motivational drive to drink under the different fluid restriction protocols, 'latency to drink' was measured on Saturday and Sunday mornings during the 16 weeks. If motivation to drink was increased on a stricter fluid restriction protocol, it would be expected that latency to approach the bottle would be shorter on the 7-day protocol than on the 5-day protocol, and that volumes consumed would be larger. A water bottle containing either the minimum allowance or 1 L of water (depending on the fluid restriction protocol) was attached to the home cage and the latency to start drinking was recorded. In circumstances where the monkey began to drink before the bottle was fully attached to the cage, the latency was scored as <1 s and given a value of 0.5 s for analysis. Since the volumes of water offered on Saturdays differed between the two protocols, an additional test was carried out, whereby the amount of fluid consumed in the first five min was also measured.

3.3 Statistical Methods

All analyses were carried out using IBM Corp. SPSS (v21, SPSS Inc, Chicago, USA) and R (R Foundation for Statistical Computing, 2015). R software was used when a suitable model was not available in SPSS, and the R packages used were as follows: glmmADMB, pscl, stringr, plyr, coda and lme4.

3.3.1 Physiological Data

All data from the physiological measures were normally distributed and analysed using a linear mixed model (LMM), with fluid restriction protocol (free access, 5-day protocol and 7-day protocol) as a fixed factor, and monkey as a random factor. For blood urea the variance of the random effect was <0.001 and so the test was performed with the random effect omitted. To compare blood results from this study to those obtained at the breeding facility, a LMM was used, with monkey colony as a fixed factor and monkey as a random factor. Finally, to compare blood results from the study to newly-restricted monkeys, a LMM was used, with fluid restriction experience (naïve or experienced) as a fixed factor and monkey as a random factor.

3.3.2 Weight Data

Weight change was assessed in three ways: over a working week, over each 4-week fluid restriction block and over a weekend. Weight change over the working week (Monday to Friday) was calculated in the following way: (Weight in kilograms Friday/Weight in kilograms Monday-1* 100). The weekly weight changes for the 5-day and 7-day protocols were normally distributed and were compared using a linear mixed model with percentage weight change as the fixed effect and monkey as the random effect. This allowed short-term weight change to be assessed. Additionally, for each fluid restriction, weekly weight changes were compared to zero (no change in weight) using a one sample *t* test. Overall weight change for a fluid restriction block (four weeks) was evaluated by taking the start and end weights of the animals and calculating percentage weight change, to evaluate longer-term effects of fluid restriction on weight. Finally, to assess the changes in weights over the weekend, percentage weight change from Friday to Monday was calculated and results from the two fluid restriction protocols were compared using a *t* test.

3.3.3 Behavioural Data

To increase the power of analyses and to detect potentially subtle changes between fluid restriction protocols, behaviours with similar functions (such as foraging, chewing and eating) were grouped together and analysed in categories (Table 2). Behaviours were assessed for differences across the three conditions (free access, 5-day protocol and 7-day protocol). Where there were differences between the three conditions, further analyses were performed to check for differences between the 5-day and 7-day protocols and whether either of these differed from free access. Certain behaviours were never seen and could not be analysed: rocking, head flicking, hand shake, self-injurious behaviour, attack and 'other' behaviours (noteworthy behaviours not defined in the ethogram; see Table 1).

Continuously-sampled behaviours occurred infrequently in the 30s scan samples, due to their rare or short nature and were therefore omitted from the scan sample data and analysed separately. As described above (section 3.2.6), drinking behaviour was captured separately as latency to approach the bottle and volume consumed in five min at weekend time points. Since animals were separated from their cage mate on

Saturdays, behavioural repertoires were not directly comparable between weekdays and weekends. Therefore, separate analyses were carried out for weekday data and Saturday data.

Behaviours were analysed by creating two models in R. The first was a LMM with an underlying gamma distribution, with monkey identity as a random effect and fluid restriction as a fixed effect. A second model omitting the effect of fluid restriction was created and an ANOVA was applied to compare the two models, to assess the overall main effect of fluid restriction (Crawley, 2005). Scan-sampled behaviours (excluding Inactivity and Pacing), all continuously sampled frequency behaviours, and self-directed behaviour were analysed in this way. Inactivity was also fitted to the above models but using an underlying Poisson distribution.

Some behaviours occurred at low frequency or were not performed by all animals and so were analysed separately. Pacing was only performed by two individuals and did not follow a normal distribution. It was therefore analysed separately for each animal using a Kruskal-Wallis test for weekdays and Mann-Whitney U test for Saturdays. Due to the low occurrence of Social behaviour and Aggression and the high prevalence of zeros in the data, these two categories were analysed using a binary logistic regression, with a random effect of monkey identity and fluid restriction as a fixed effect.

Table 2. Categories of behaviours used for statistical analysis.

Category	Included Behaviours
Scan Sampled (every 30s)	
Inactivity	Alert, Not Alert, Hunched
Consumption	Eating, Chewing, Foraging
Interact	Interact with physical environment – hands/feet, Interact with physical environment - oral
Locomotion	Relaxed Locomotion, Agitated Locomotion
Allogroom	Allogroom - donor, Allogroom - recipient
Self-groom	Self-groom
Pacing	Locomotor stereotypy
Continuously Sampled (Duration)	
Aggression	Aggression to cage mate, Aggression directed outside cage
Social	Affiliative, Being mounted, Dominance, Mounting, Play with cage mate, Submissive to cage mate
Self-directed	Self-scratching, eye rub
Continuously Sampled (Frequency)	
Body Shake	Body Shake
Yawn	Yawn
Abnormal	Abnormal

3.3.4 Water Bottle Approach and Consumption

Latencies to approach the bottles were not normally distributed and were analysed using a Mann-Whitney *U* test. In order to make Saturday consumption data comparable across the monkeys, volumes drunk were converted to a percentage of each animal's minimum daily allowance. These data were not normally distributed and were analysed using a Mann-Whitney *U* test.

3.3.5 Task Performance

Monkey 1 was excluded from the task performance analysis (i.e. the number of trials performed on work days as a function of fluid restriction protocol). This was due to the difficulty of his task increasing across the study, as was necessary for the electrophysiological data collection, and the varied setting in which he worked (electrophysiology laboratory and MRI scanner). Trial data for the remaining three monkeys were not normally distributed and differences in the number of trials performed when on the 5- and 7-day fluid restriction protocols were assessed using a Mann-Whitney test for each monkey individually. To assess the effect of weekend water intake on Mondays work performance, a Pearson correlation was calculated using percentage weight change from Friday to Monday and the number of trials performed on a Monday. In addition, trials performed on Monday were compared to 1000 (an about acceptable laboratory performance) using a one-sample Wilcoxon sign rank test.

3.4 Results

3.4.1 Fluid Intake of Individual Animals

The four animals differed in their free access intakes (FAI) and in the daily minimum fluid allowance established to ensure adequate work-rates (Table 3; see Chapter 2, section 2.3 for details on fluid allowance calculations).

Table 3. The free access intake (FAI) of each monkey and his daily minimum fluid allowance in total ml, %FAI and in ml/kg/day.

Monkey	Free Access Intake (ml)	Fluid Allowance (ml)	% FAI	ml/kg/day
1	645	200	31	15
2	880	150	17	14
3	910	355	39	26
4	305	150	49	17

3.4.2 Physiological Measures

There were no significant effects of restriction protocol type on physiological blood measures for the four monkeys. Concentrations of sodium (Na), haematocrit (HCT), urea and creatinine (Cr) in the blood did not differ across the 5-day and 7-day protocols and the free access period (LMM: Na, HCT, Cr: $F_{(2,18)} < 2.98$, $p > 0.076$; Urea: $F_{(2,21)} = 0.89$, $p = 0.42$; Figure 1). However, urine measures of osmolality (Osm), creatinine (Cr) and specific gravity (SG) significantly differed across conditions (Osm: $F_{(2,11)} = 16.91$, $p < 0.001$; Cr: $F_{(2, 9.98)} = 7.31$, $p = 0.0011$; SG: $F_{(2, 9.98)} = 24.30$, $p < 0.001$; Figure 2). All three urine measures were lower when monkeys had free access to water than during either the 5-day or 7-day protocols (Bonferonni *post hoc* comparisons all $p < 0.05$; Table 4), but there was no difference between the two restriction protocols (Bonferonni *post hoc* comparisons all $p > 0.54$; Table 4).

Table 4. Bonferonni *post hoc* pairwise comparisons of the effect of fluid restrictions (free access, 5-day, 7-day) on urine measures of hydration.

Measure	(I) Fluid restriction	(J) Fluid restriction	Mean Difference (I-J)	SEM	df	*p* - value
Osmolality (mOsmol/kg)	Free Access	5-day	-1006.81	143.46	10.88	<0.001
	Free Access	7-day	-869.63	151.66	10.62	<0.001
	5-day	7-day	137.19	154.82	12.31	1.00
Creatinine (mmol/L)	Free Access	5-day	-28.14	8.11	12.44	0.01
	Free Access	7-day	-30.60	8.06	11.70	0.008
	5-day	7-day	-2.46	8.11	12.44	1.00
Specific Gravity	Free Access	5-day	-0.03	.004	11.88	<0.001
	Free Access	7-day	-0.03	.004	11.36	<0.001
	5-day	7-day	0.006	.004	11.88	0.54

There were some differences in the physiological measures taken from blood samples between the experimental monkeys and those at the breeding centre (Figure 1). Levels of urea were higher at the CfM breeding centre (Mean difference: 1.08 mmol/L, $F_{(1,40)} = 8.36$, $p = 0.006$), whilst creatinine levels were lower (Mean difference: 15.75 µmol/L, $F_{(1,11.08)} = 5.79$, $p = 0.035$). The remaining blood measures did not differ between males in the two colonies (Na: $F_{(1,12.99)} = 0.004$, $p = 0.95$; HCT: $F_{(1,8.78)} = 4.60$; $p = 0.06$). Taken together, the physiological data suggest that fluid restriction protocols as used here have no negative impact on blood physiology in male macaques.

To assess the initial effects of fluid restriction, the four monkeys involved in this study were compared with two other monkeys in the Newcastle colony who were newly subjected to fluid restriction. Since there was no difference in blood measures between the free access, 5-day and 7-day protocols, these data were pooled and compared to all blood taken from the newly restricted monkeys. There were no differences in any of the blood measures (LMM: Na mean difference = -0.88 mmol/L, $F_{(1,4.9)} = 0.27$, $p = 0.63$; HCT mean difference = -1.69%, $F_{(1,4.9)} = 0.77$, $p = 0.42$; Cr mean difference = -19.34 µmol/L, $F_{(1,4.9)} = 3.38$, $p = 0.14$ and Urea mean difference = 0.48, $F_{(1,4.9)} = 0.78.$, $p = 0.41$; Figure 1).

Finally, the post mortem reports from the kidneys of two deceased monkeys from Newcastle stated that: "(The kidneys) exhibit minimal chronic interstitial multifocal

nephritis, a non-specific finding, which likely represents an incidental finding in this case" and for the second monkey: "Both kidneys appear well organised. There is no evidence of extensive mineralisation within the kidney and no material suggestive of uroliths is observed within the renal pelvis. There are rare small foci of inflammation and interstitial fibrosis which would not have been of clinical significance." Although the monkeys were not included in the data collection for this study, these reports suggest a 5-day fluid restriction experienced by the monkeys did not negatively impact on their kidney organisation or function.

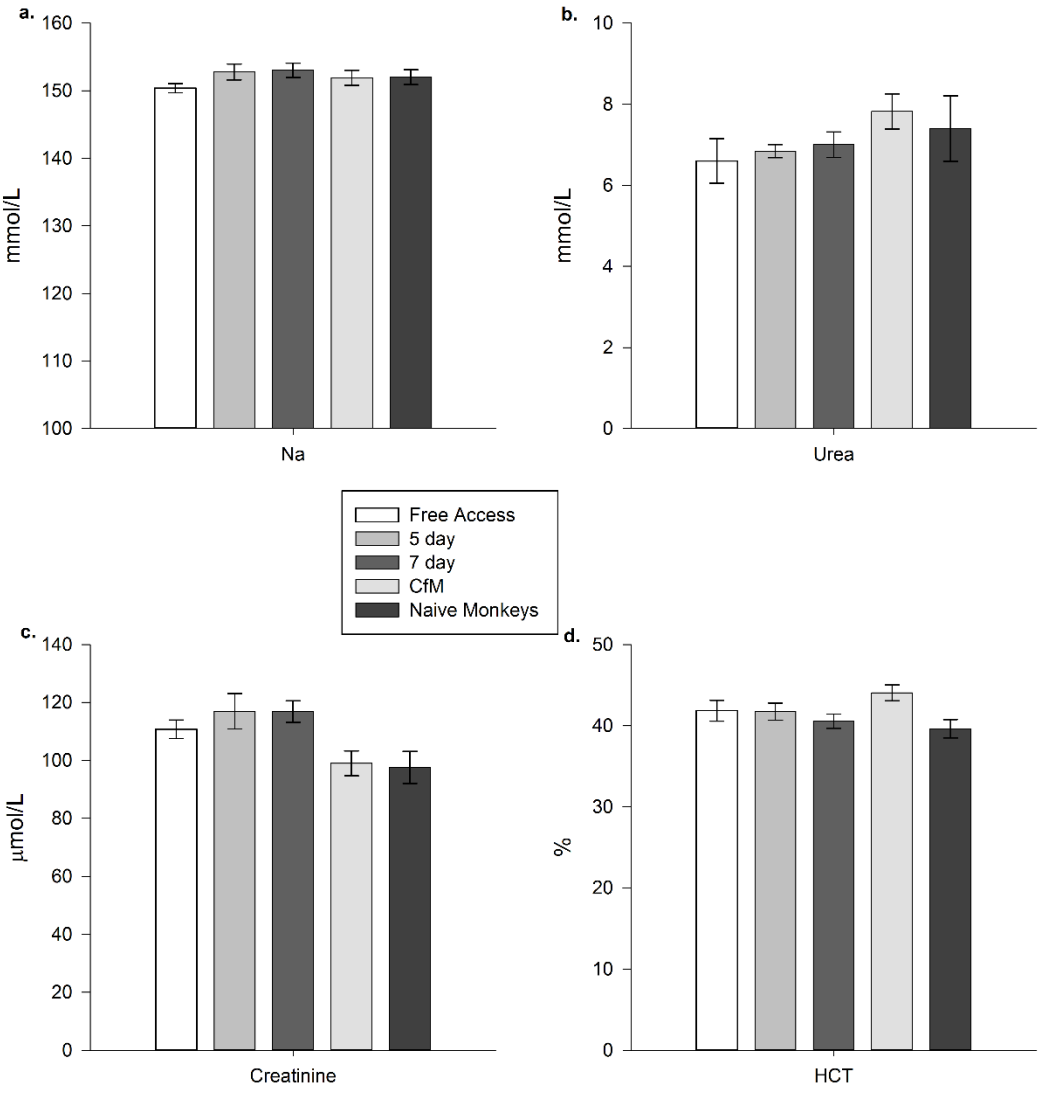


Figure 1. The effect of fluid restriction protocols on blood measures of hydration (mean ± Standard Error of Mean, SEM) in monkeys recruited for this study (free access, 5-day, 7-day: N = 4), monkeys with *ad libitum* access to water at the breeding facility (CfM: N = 13) and newly restricted monkeys (Naïve monkeys: N = 2) for: (a) sodium; (b) urea; (c) creatinine; and (d) haematocrit.

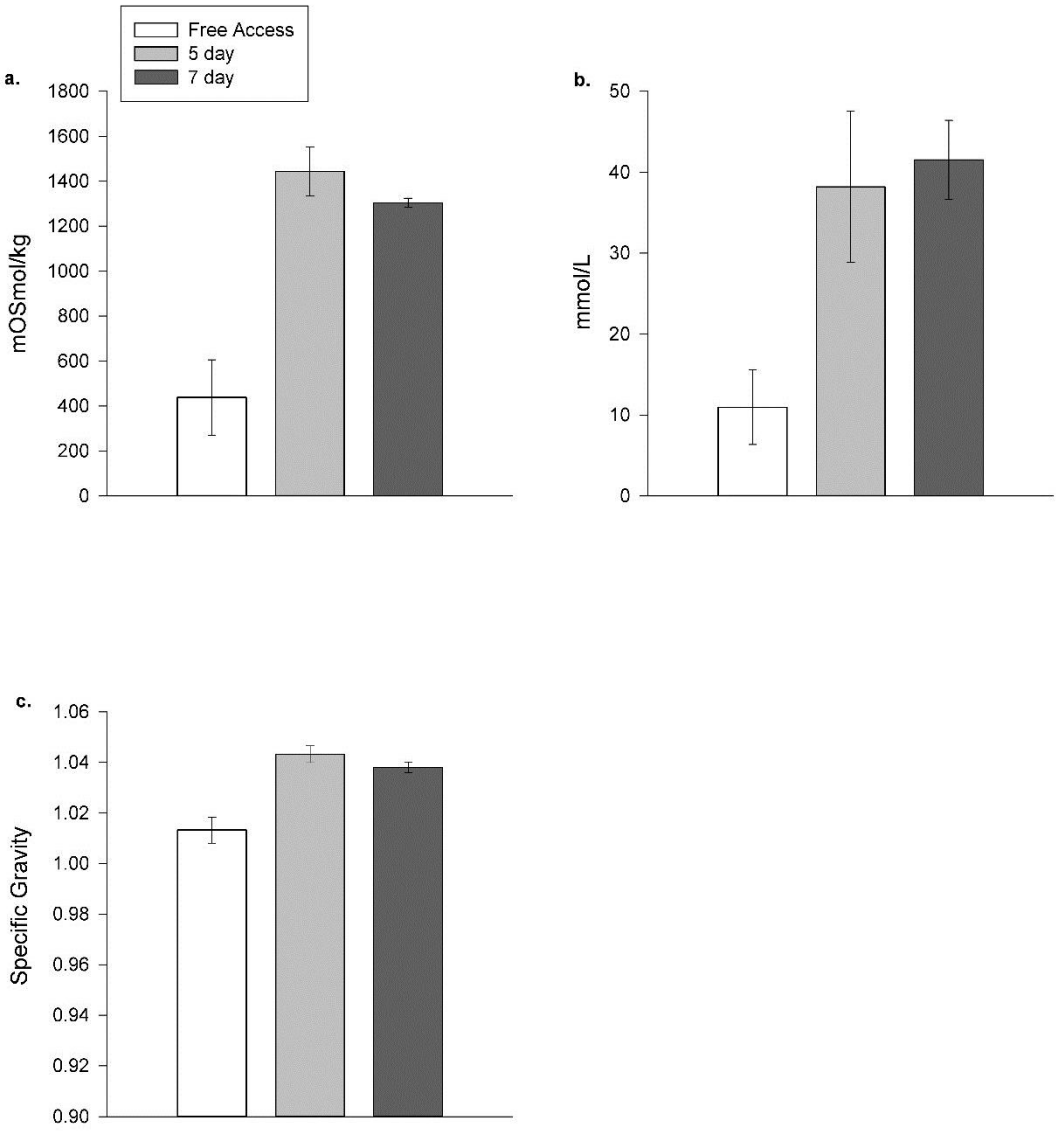


Figure 2. The effect of fluid restriction protocols on urine measures of hydration (mean ± SEM) during free access, the 5-day protocol and the 7-day protocol for: (a) osmolality; (b) creatinine and; (c) specific gravity

3.4.3 Weight

To investigate possible weight loss associated with fluid restriction, daily changes in body weight were measured throughout the working week, across the 16 weeks of fluid restriction protocols. From Monday to Friday, weight loss occurred on the 5-day protocol but not on the 7-day protocol (LMM: $F_{(1,57.20)} = 9.48$, $p = 0.003$; Figure 3). On average, monkeys lost body mass (mean weight change = -0.95% body mass) during the week on the 5-day protocol (one sample *t* test, test value = 0, $t_{(29)} = 3.39$ $p = 0.002$), whilst their body mass remained relatively constant (mean weight change = + 0.10%) on the 7-day protocol ($t_{(31)} = 0.45$, $p > 0.66$). However, across a fluid restriction block (4 weeks), there were hints of an opposite trend, with animals maintaining weight on the 5-day protocol (mean weight change = -0.16%), but losing weight on the 7-day protocol (mean weight change = -1.66%); Appendix A, Figure 1. However, neither change across a four-week block was significantly different from zero (one sample *t* test, test value = 0, 5-day: $t_{(7)} = 0.21$, $p = 0.81$; 7-day: $t_{(7)} = 2.18$, $p = 0.066$), nor were they different from one another (LMM: $F_{(1,11)} = 2.12$, $p = 0.17$). Therefore, weight change was not consistent across the two fluid restriction protocols, with shorter-term weekly changes in weight on the 5-day protocol, but no significant longer term (4-week) changes in either of the two protocols. This suggests that weight loss is not a major concern for animals on fluid restriction protocols, at least over a 16-week period.

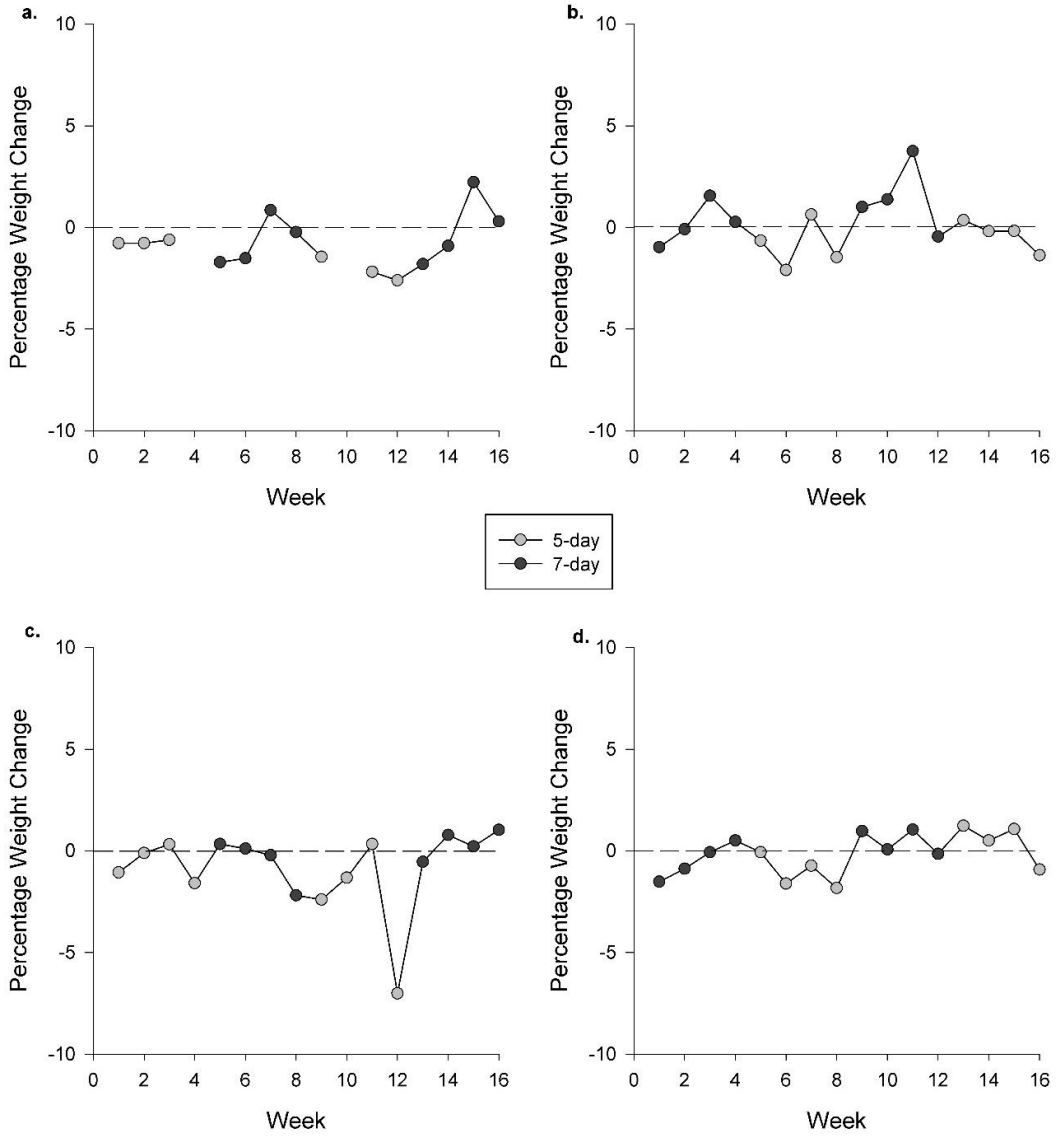


Figure 3. The weekly percentage weight change calculated from the beginning of each fluid restriction block (weight in kilograms Friday/Weight in kilograms Monday) -1*100): (a) Monkey 1; (b) Monkey 2; (c) Monkey 3; (d) Monkey 4. Dashed lines indicate no change in weight.

3.4.4 Behavioural Measures

In addition to physiological and morphological measures, the monkeys were also filmed in their home cages to assess whether the fluid restriction protocols caused changes in behaviour. On weekdays, significant differences were found in the frequency of nine behaviours occurring across the free access versus fluid restriction protocols (Figure 4 a-e). These were: Interaction (χ^2 (2) = 42.27, $p < 0.001$), Locomotion (χ^2 (2) = 11.77, $p = 0.0027$), Self-groom (χ^2 (2) = 37.35, $p < 0.001$), Body shake (χ^2 (2) = 30.86, $p < 0.001$), Yawn (χ^2 (2) = 101.32, $p < 0.001$), Self-directed (χ^2 (2) = 17.09, $p < 0.001$), Abnormal (χ^2 (2) = 10.07, $p = 0.0065$), Social (χ^2 (2) = 8.72, $p = 0.013$), and Inactivity (χ^2 (2) = 6.51, $p = 0.039$) (Figure 4a-e). For six of these behaviours (Interaction, Locomotion, Self-groom, Body shake, Yawn and Self-directed), the frequency was lower in the 5-day and 7-day protocols compared to free access (5-day: all $t_{(184)} < 7.06$, $p < 0.006$; 7-day: all $t_{(194)} < 7.69$, $p < 0.001$), with no difference in frequency between the two fluid control protocols (all $t_{(198)} < 1.07$, $p > 0.28$).

Three out of 13 behavioural categories differed between the two fluid control protocols. Abnormal behaviour was lower in frequency in the 5-day protocol compared to free access ($t_{(184)} = 2.68$, $p < 0.001$) and the 7-day protocol ($t_{(198)} = 2.79$, $p = 0.005$), but there was no difference between free access and the 7-day protocol ($t_{(194)} = 0.08$, $p = 0.94$). However, inactivity was lower on the 7-day protocol compared with free access ($t_{(194)} = 2.55$, $p = 0.01$), but not different to the 5-day protocol ($t_{(198)} = 1.39$, $p = 0.166$). There was also no difference between free access and the 5-day protocol ($t_{(184)} = 1.18$, $p = 0.24$). Social behaviour was lower on the 7-day protocol than on the 5-day protocol ($t_{(198)} = 2.13$, $p = 0.033$) and the free access protocol ($t_{(194)} = 0.28$, $p = 0.005$), but there was no difference between free access and the 5-day protocol ($t_{(184)} = 0.76$, $p = 0.45$).

No other behaviours were affected by fluid restriction (Allogroom, Consumption [foraging, eating and chewing] χ^2 (2) < 2.99; Aggression χ^2 (1) = 1.08; Pacing, $H_2 < 3.36$; all $p > 0.16$; Figure 4 a, b, e). Overall, results showed no consistent pattern of fluid restriction changing behaviour in line with impoverished welfare.

On Saturdays, monkeys were separated from their cage mates, and their behaviours were not comparable to behaviours performed during the free access periods or other

weekdays when cage mates had been present. Therefore, behavioural data collected on Saturdays were only compared with that from other Saturdays. On Saturdays, there was a significant effect of fluid restriction on two behaviours. The first was consumption (foraging, chewing and eating), which was lower when animals were on the 7-day protocol compared to the 5-day protocol (χ^2 (1) = 8.68, p = 0.003). The second behaviour was pacing, which was only sufficiently frequent to allow for quantitative analysis in two of the four animals. Pacing increased for one monkey on the 7-day protocol compared to the 5-day protocol (U = 110, z = 2.43, p = 0.026; Figure 5b), whilst the second monkey showed no change in pacing behaviour (U = 107, z = 1.58, p = 0.123; Figure 5b). All remaining behaviours showed no difference in frequency between 5-day and 7-day protocols (Interaction, Locomotion, Self-Groom, Inactivity, Body Shake, Yawn, Abnormal, Self-Directed, Aggression and Social, χ^2 (1) < 3.23, p > 0.07 for all; Figure 5a, c, d, e). In summary, only two behaviours differed over the weekend between the two fluid restriction protocols.

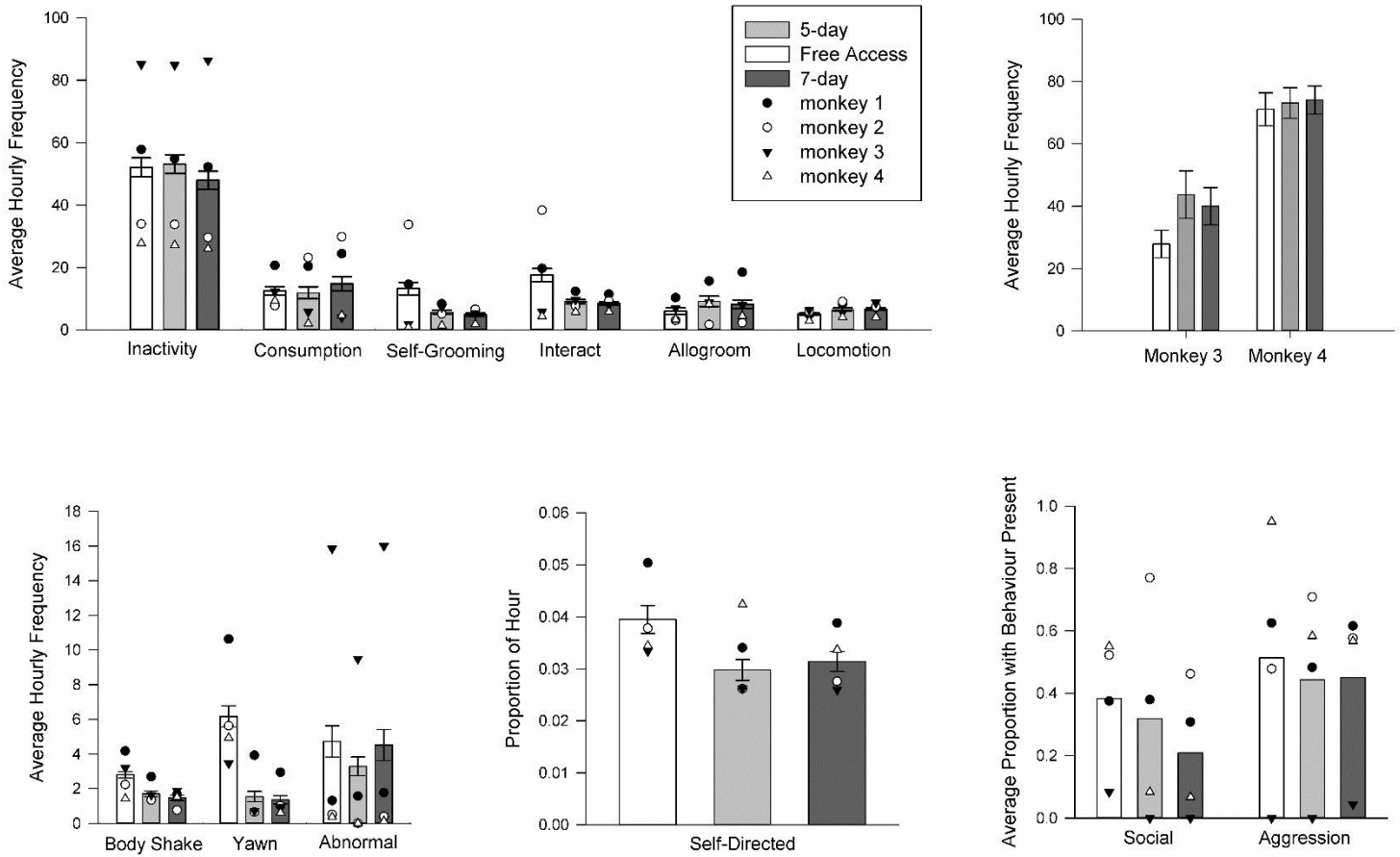


Figure 4. The effect of free access to water, 5-day and 7-day fluid restriction protocols on behaviours performed on weekdays. Behaviours are grouped by the sampling methods used: (a) scan sampled behaviours; (b) scan sampled pacing frequency for Monkeys 3 and 4; (c) continuously sampled, frequency-only behaviours; (d) continuously sampled duration of scratching behaviour; (e) continuously sampled behaviours (binary data) with a high prevalence of zero. The means for individual monkeys are denoted by overlaid symbols.

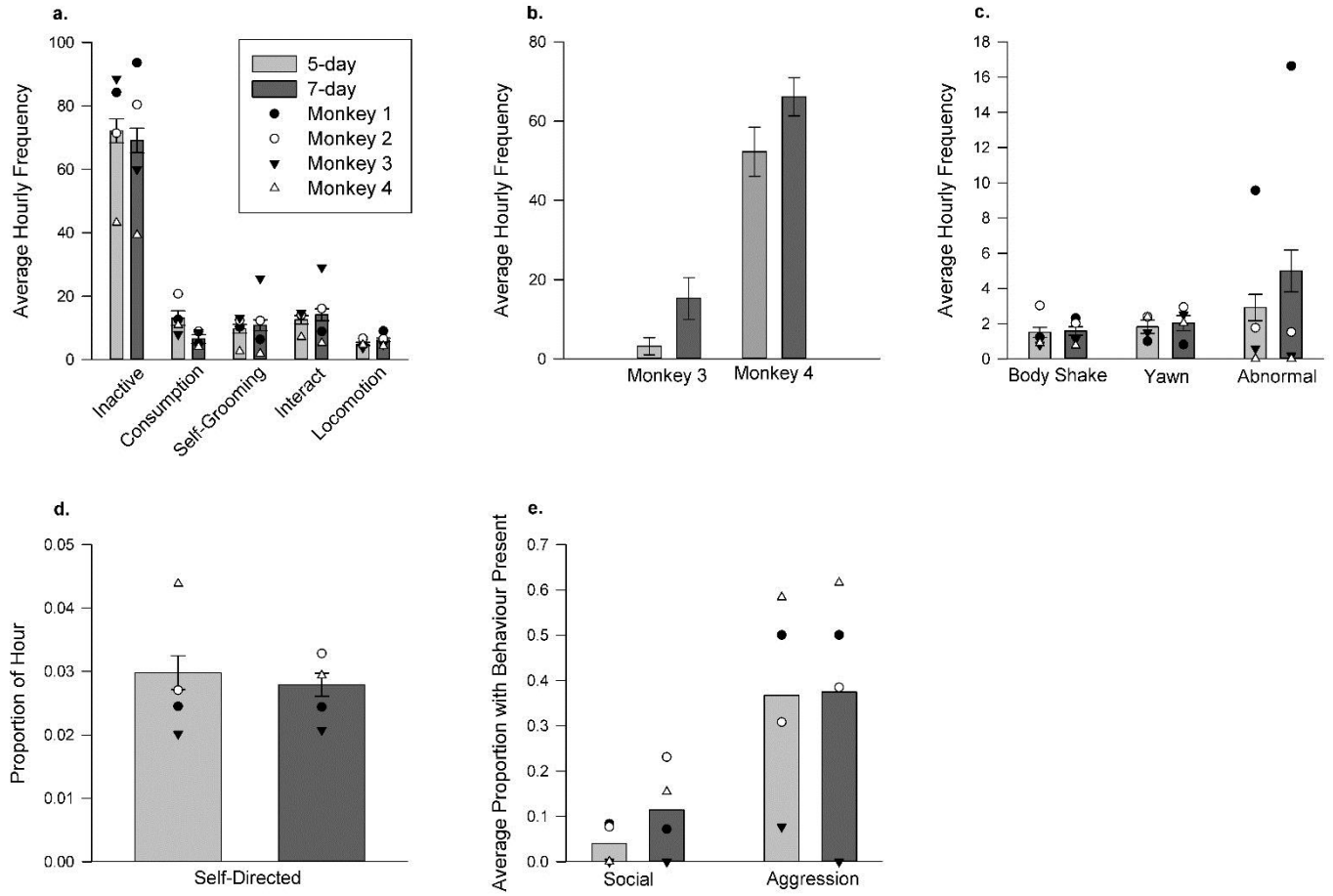


Figure 5. The effect of 5-day and 7-day fluid restriction on behaviours performed on Saturdays. Behaviours are grouped by the sampling methods used: (a) scan sampled behaviours; (b) scan sampled pacing frequency for Monkeys 3 and 4;(c) continuously sampled, frequency-only behaviours; (d) continuously sampled duration of scratching behaviour; (e) continuously sampled behaviours (binary data) with a high prevalence of zeros. The means for individual monkeys are denoted by overlaid symbols.

3.4.5 Water Bottle Approach and Consumption

On Saturdays, the monkeys approached the water bottles attached to their home cages significantly quicker on the 7-day protocol (Median = 2 s), than on the 5-day protocol (Median = 4 s) (Mann Whitney, $U = 2.24$, $p = 0.03$; Appendix A, Figure 2a). The monkeys also drank more in five min on Saturdays on the 7-day protocol (Median percentage of fluid allowance consumed = 100%), compared to the 5-day protocol (Median percentage of fluid allowance consumed = 50%), (Mann Whitney, $U = 3.28$, $p = 0.001$; Appendix A, Figure 2c). There was no effect of fluid restriction protocol on the latency to approach the water bottle on Sundays (Mann Whitney, $U = 0.46$, $p = 0.647$; Appendix A, Figure 2b). Thus motivation to drink was increased on Saturdays on the 7-day protocol.

3.4.6 Task performance

Only three monkeys were included in the analysis of task performance (one monkey had regularly changing task demands, required by the experimental design, which precluded this specific analysis). There was no overall increase in the daily numbers of trials performed in their respective cognitive tasks when they were subjected to the 7-day protocol, rather than the 5-day protocol (Mann Whitney, $U < 1.44$, $p > 0.15$ for all; Figure 6). The performance on Monday is of particular importance, since animals often do not perform enough trials for scientific data to be collected on the 5-day protocol. On Mondays, there was a significant correlation between the percentage weight change over the weekend (from Friday to Monday) and the number of trials performed: when weight decreased over the weekend, more trials were performed on the Monday (Pearson correlation, $R^2 = - 0.49$, $p < 0.01$; Figure 7). Weight change over the weekend differed between the two fluid restrictions (t test, $t_{(28)} = 3.58$, $p = 0.001$). On average, monkeys gained 0.83% weight over the weekend on the 5-day fluid restriction and performed fewer than 1000 trials on Mondays (Median = 686, test value = 1000, W = 3.64, $p < 0.001$). Conversely, on the 7-day fluid restriction, monkeys lost 0.76% body mass and completed an average of 981 trials on Mondays (test value = 1000, W = 0.065, $p = 0.95$). Slight weight loss over the weekend on the 5-day protocol resulted in poor performance on a Monday, whereas monkeys were more motivated to work on a Monday on the 7-day protocol without free access to water over the weekend.

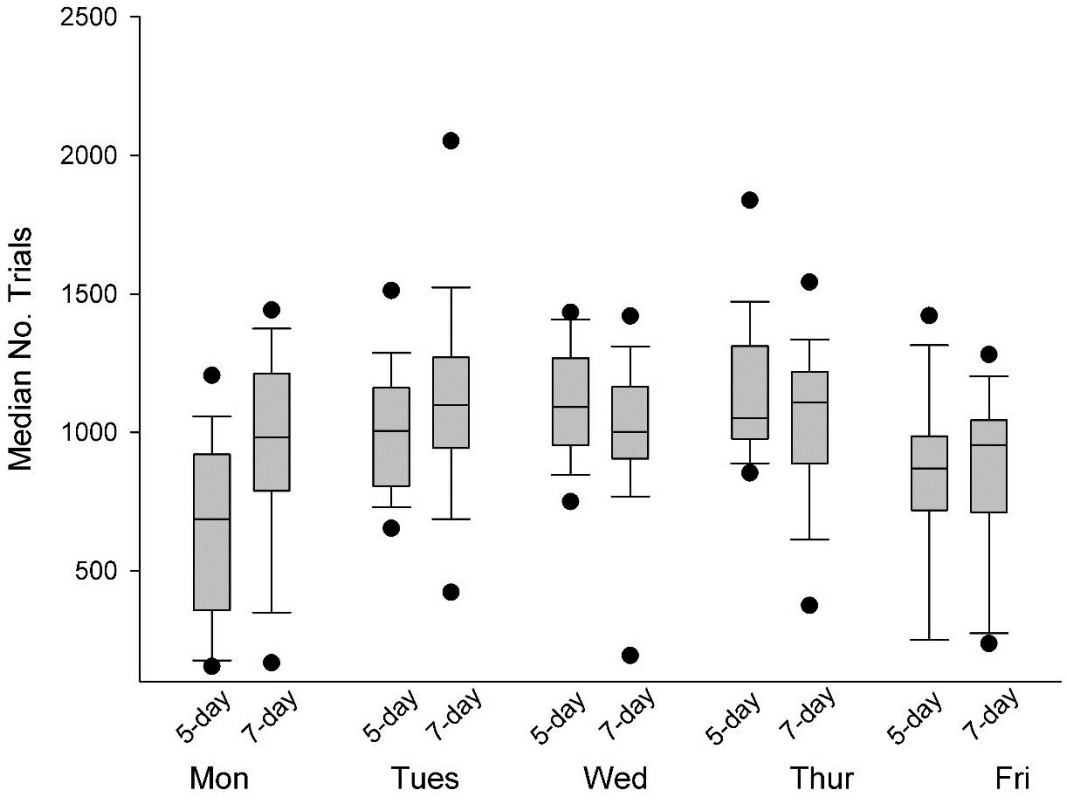


Figure 6. Median number of trials performed daily, averaged across all monkeys (N = 3). Filled circles represent 5th and 95th percentiles.

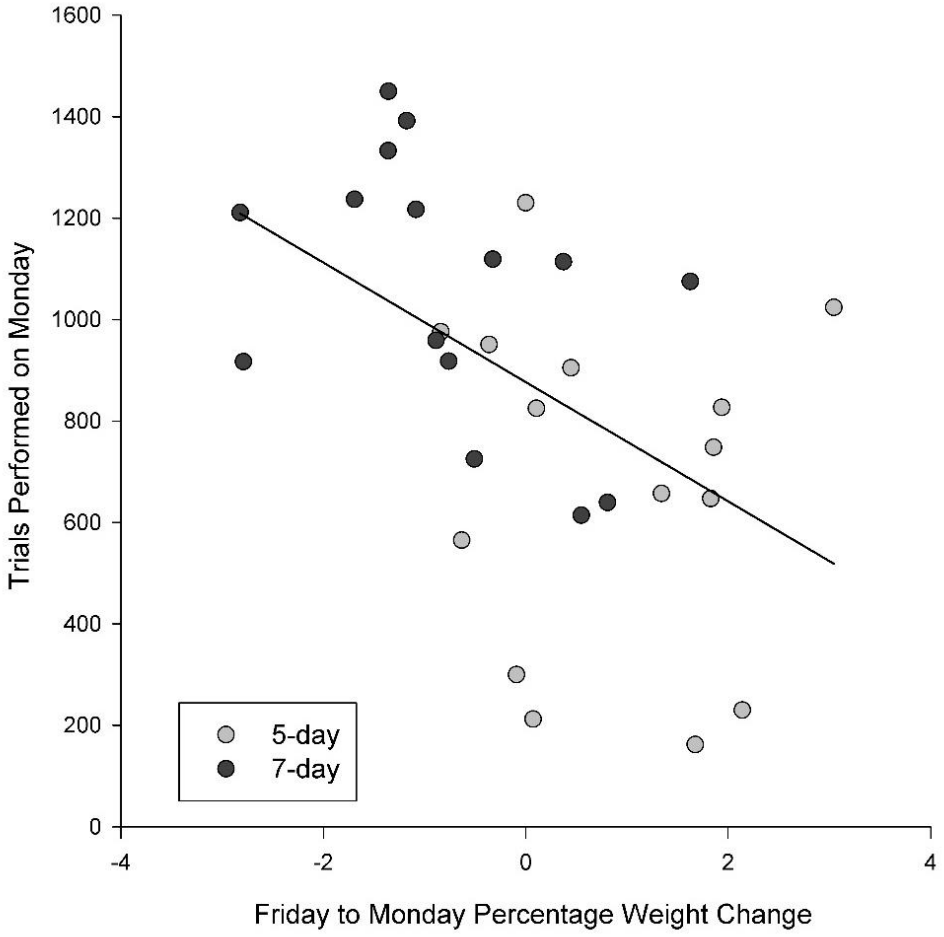


Figure 7. The effect of Friday to Monday percentage weight change on the number of trials performed on a Monday

3.5 Discussion

This study provides the first objective and quantitative data on the effects of fluid restriction protocols on the physiology, behaviour and performance of laboratory macaques used in behavioural neuroscience. Given the range of data analysed, each of the different measures is discussed in turn.

3.5.1 Physiological Changes

One primary concern with fluid restriction protocols is that they dehydrate the animals, leading to reduced welfare and poor animal condition (Prescott et al., 2010). However, I found that blood measures of hydration were the same across all three conditions (5-day protocol, 7-day protocol and free access), and were within ranges observed across other rhesus macaque facilities where animals are on constant *ad libitum* fluid access (Table 5). However, since the values obtained in other facilities include animals of differing ages compared to our males, the blood measures were also compared with samples acquired from a sample of similarly aged male monkeys at CfM, which had never experienced any fluid restriction protocol. Two blood measures did differ slightly between our monkeys and those at CfM: CfM's macaques had higher levels of urea and lower levels of creatinine. However, these do not immediately point to long-term effects of fluid protocol use in our animals: the higher levels of urea were the opposite of what would be expected for animals with *ad libitum* access to water, and values for both colonies still lie within normal ranges (Table 5). Furthermore, when compared with two monkeys experiencing fluid restriction for the first time (previously naïve), there was no difference in blood measures between them and the experimental animals.

In addition, I found that urine was more concentrated for both fluid restriction protocols compared to the free access periods, and there were no differences between the two fluid restriction protocols. Taken together, these results suggest that macaques can cope with a reduced fluid intake from when they first experience fluid restriction protocols, that there appears to be no long term damage of fluid restriction: overall, the monkeys' kidneys were well functioning and efficiently retained fluids when access to water was limited. Their ability to efficiently retain fluids may be an adaptation to seasonal rainfall and periods of restricted water access in their natural environment

(Lindburg, 1977). It is important to highlight that all four macaques used in this study had been previously water restricted on the 5-day protocol for over four years, and yet their physiological responses to fluid restriction remained normal. In addition, the post mortem examination of two deceased monkeys from Newcastle reported good kidney organisation. These monkeys had been fluid restricted on the 5-day protocol for 5 and 11 years and experienced no damage to their kidney structure. This suggests that a 5-day protocol has no negative physiological effects on a long-term basis, arguing against the concern that keeping animals on fluid restriction protocols for long periods may cause physiological harm (Prescott et al., 2010).

Overall, the physiological measures suggest that there is no short-term welfare impact on being on either protocol over a four-week period, and no significant difference between the two. Whilst the data also suggest that no long-term harm is caused by monkeys being repeatedly subject to periods of 5-day fluid restriction, it is not certain whether this is the case for the 7-day fluid restriction protocol, as the 7-day fluid restriction protocol has not been implemented for extended periods of time. Further long-term studies would be required to investigate this.

3.5.2 Weight Change

Potential weight loss is a key welfare issue surrounding fluid restriction, with concerns that fluid restriction and the potential associated reduction in food intake (Cizek and Nocenti 1965; Collier and Levitsky 1967; Engell 1988) could lead to a substantial loss in body mass (Prescott et al., 2010). Within a working week (Monday-Friday), weight loss occurred on the 5-day protocol but not on the 7-day protocol. However, across a fluid restriction block, the opposite effect was found, with animals maintaining weight over the 5-day protocol, but not on the 7-day fluid restriction protocol, where there was a small degree of weight loss (around 2% over a four-week period). Although these results initially appear contradictory, they can be explained by weight changes over the weekend. When on the 5-day protocol, monkeys tended to gain weight on free access to water, thus starting the week at a higher mass (mean weight change = +0.83%). In contrast, without the opportunity to work beyond their minimum on weekend days, monkeys on the 7-day protocol tended to lose weight over a weekend (mean weight change = -0.76%), resulting in a slight weight loss over the 4-

week block. Whether weight loss would continue on an extended 7-day protocol is impossible to say from my data; it requires further longer-term research. However, the data are conclusive in showing that a 5-day protocol does not lead to excessive weight loss, or indeed any weight loss, and a 7-day restriction regime over the course of 4 weeks induces no statistically significant weight loss, nor any rapid or sustained weight loss that would raise any immediate welfare concern.

3.5.3 Behaviour

There were some behavioural changes in the monkeys between the free access and fluid restriction conditions. Whilst some behavioural changes may be indicative of reduced welfare during the two fluid restriction protocols, for example, increased stereotypic pacing in one animal (Gottlieb et al., 2015), others suggest the opposite; that the monkeys' welfare was compromised more during the control period. Body shaking, self-grooming and yawning are considered to be indicative of anxiety in macaques (Ninan et al., 1982; Deputte, 1994; Schino et al., 1996; Major et al., 2009), making it surprising that these behaviours were more prevalent in the free access period compared to during either fluid protocol. One possible reason for this observation was that the free access data were collected over the Christmas break, when animals were not working in experiments and had free-access to water. Collecting free access data during breaks was necessary because fluid restriction and working routines are intrinsically linked. Fluid restriction is only permitted when the monkeys have the opportunity to earn fluid in the laboratory, and running animals in experiments on free access is not possible. However, this meant there were also changes to laboratory and husbandry routines: monkeys did not take part in experimental procedures, had reduced social contact with humans (research and animal care staff), and husbandry routines were different to those experienced during a typical experimental week. Although animals may experience similar periods throughout the year (e.g. holiday weekends, and festive breaks), these changes in routine could potentially increase anxiety related behaviours in the free access period (reviewed by Bassett and Buchanan-Smith, 2007). Therefore, it is difficult to know if behavioural differences between free access and fluid restriction protocols were due to fluid access, changes in routine, or a combination of the two. When husbandry and daily routine return to normal, the corresponding decrease in anxiety could theoretically mask an increase in anxiety from

fluid restriction. Despite this potential confound, it can be safely concluded that fluid restriction does not increase anxiety more than a change in husbandry regime, if any.

There were also very few behavioural differences observed between the two fluid restriction protocols, and again, the results were not consistent. For example, on weekdays, abnormal behaviours were higher on the 7-day fluid restriction, potentially indicating increased stress levels (Lutz et al., 2003). However, inactivity was lower on the 7-day fluid restriction, which is generally indicative of improved welfare (Lutz and Novak 2005; Baker et al. 2014). It is surprising that inactivity decreased in monkeys subjected to a stricter fluid restriction, since studies on humans have documented an increase in fatigue when subjects are fluid-deprived, with participants anecdotally reporting decreased activity levels (Pross et al., 2014). Decreasing inactivity levels (sometimes indicative of improved welfare) occurred alongside increases in abnormal activity, making it impossible to identify any clear impacts on welfare from the 7-day protocol.

Small behavioural differences between protocols were also observed on Saturdays. Consumption (foraging, eating and chewing) was lower on the 7-day protocol compared to the 5-day protocol. There are two possible explanations for this. One possible explanation is that because water is required to absorb and digest food, animals cannot eat as much on the 7-day protocol compared to the 5-day protocol. This voluntary reduction in consumption has been previously documented in rats and humans (Cizek and Nocenti, 1965; Collier and Levitsky, 1967; Engell, 1988), and is one of the concerns surrounding fluid restriction (Prescott et al., 2010). Alternatively, it may not be that the animals are under-eating on the 7-day protocol, but rather that they are over-eating on the 5-day protocol: “bingeing” can occur when monkeys are given free access to water on the 5-day regime (Toth and Gardiner, 2000). Both of these explanations are supported by changes in weight over the weekend, with increases on the 5-day but decreases on the 7-day protocols, making it difficult to tease apart the two. Overall, regardless of what causes the difference in consumption behaviour at weekends, it should be noted that these changes were not of a magnitude to cause weight loss of concern in our monkeys.

The second change was in pacing behaviour. Two of the animals in our group performed pacing behaviour during all protocols. In one of the two, higher levels of pacing occurred over the weekend on the 7-day compared with the 5-day protocol. Stereotypies in captive macaques are often used as indicators of suboptimal welfare and may indicate higher levels of stress in this individual (Novak et al., 2006). However, their prevalence alone should not be relied upon as a single measure of wellbeing (Mason and Latham, 2004), and data from one animal remain too limited to enable a firm conclusion. In addition, stereotypies can be interpreted as a coping behaviour (Mason and Latham, 2004; Novak et al., 2006) and as such, animals performing these behaviours may experience a more positive state of wellbeing than is often assumed. It is therefore difficult to draw definite conclusions from these data.

3.5.4 Water Bottle Approach and Consumption

When given access to water on Saturdays, monkeys appeared more motivated to drink on the 7-day protocol than on the 5-day protocol: they approached the bottle more quickly and consumed a larger volume of water. This may be due to many reasons, including a dryness or unpleasant taste in the mouth, as shown in humans (Rolls et al., 1980). However, it is impossible to infer the subjective experience (e.g. thirst) of the animals from our data. Therefore, being on a 7-day protocol increased the animals' motivation to drink, but it is uncertain what state caused this change in motivation.

3.5.5 Task Performance

An important aspect of this study was to assess the scientific outcomes associated with the use of different fluid restriction protocols. Typically, on a 5-day fluid restriction protocol, animals do not participate in a sufficient number of trials to collect a robust data set (around 1000 trials are required per day for these particular tasks). Consequently, data collection is not usually attempted on a Monday. The number of trials performed on a Monday in this study were too low on the 5-day fluid restriction regime to attempt electrophysiological recordings, given the scientific requirements of the studies involved. The most likely reason for this is that monkeys were not motivated to drink after increased access to water over the weekend. However, when the monkeys were restricted over the weekend on the 7-day protocol, performance on Mondays increased to levels that would generally allow electrophysiology to be performed. This

suggests that a 7-day fluid restriction protocol might enable scientific studies to be conducted five days per week (or seven days, if recording continued over the weekend), which could significantly reduce the duration of a study by at least 20%. This would mean that the time individual monkeys spend on a fluid restriction regime would be similarly reduced.

3.6 Conclusions

This study addressed the need for scientific data on the impact of different fluid restriction protocols on the welfare and performance of laboratory primates used in neuroscience research (Prescott et al., 2010). The use of fluid restriction protocols are contentious (Orlans, 1991; Willems, 2009; Westlund, 2012) and it is crucial that we better understand how they affect experimental animals in order to make more informed decisions about their use. The main conclusions are that:

1. Male macaques physiologically cope with periods of fluid restriction, maintaining blood parameters within normal ranges by concentrating their urine in response to both protocols. There were no detectable short-term effects of either the 5-day or 7-day protocol, or any long-term (> 4 years) effect of a 5-day protocol, on kidney function. Further work is required to establish whether the same results would be seen in female macaques.
2. There were relatively small changes in behaviour detected by in-depth analysis, with some behaviours indicative of poor welfare being associated with fluid restriction protocols, and others with free access to water.
3. 5-day and 7-day fluid restriction protocols do not lead to rapid and sustained weight loss that would be of immediate welfare concern. More data are required to assess the long-term impact of 7-day fluid restriction on weight changes.
4. Animals are more motivated to drink in their home cage when on a 7-day protocol compared to 5-day, but the subjective experiences of the animals are unknown.
5. Improved task performance on a 7-day protocol compared to a 5-day protocol could allow more rapid collection of sufficient scientific data, and reduced time spent on fluid restriction protocols for experimental animals.

These data mostly fail to show the significant detrimental effects on the welfare of laboratory macaques, which often have been predicted to arise from the use of fluid restriction protocols. This study counters and alleviates many of the widely-held welfare concerns surrounding these methods.

Table 5. Values of published rhesus macaque blood parameters

Source	Age	N	Na		Urea		Creatinine		HCT	
			mmol/L		mmol/L		µmol/L		%	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lee et al., 2012	2-5	29	145.68	3.68	5.91	1.59	66.3	15.91	34.87	4.49
Hassimoto et al., 2004	3.5	6	139	5	9.28	1.43	41.55	6.19	43.4	4.7
Chen et al., 2009	3-5	18	149.71	3.07	8.47	1.21	69.73	11.51	43	0.02
Ribeiro Andrade et al., 2004	3.5-16	21	-	-	11.13	3.71	-	-	37.55	3.23
Primate Ageing Database (Indoor housing)	4-15	57- 157	148.88	11.48	6.40	2.98	111.65	71.07	41.04	10.35
Primate Ageing Database (Indoor housing)	8-9	3 - 44	150.33	1.7	6.14	2.01	122.35	18.39	40.42	3.58
Primate Ageing Database (All housing)	4-15	62 - 192	146.382	9.00	6.81	3.38	109.09	67.80	41.04	9.88
Primate Ageing Database (All housing)	8-9	15 - 53	145.5	8.55	6.86	2.59	112.62	20.69	40.62	3.42
Buchl and Howard, 1997	3-4	30	148	3	6.43	1.07	79.46	8.84	-	-
Levine, 1995	3-7	-	145	1.5	7.14	1.07	83.98	11.05	-	-

Chapter 4: The Use of Preferred Fluid Rewards to Refine Fluid Restriction Protocols

4.1 Introduction

The first chapter of this thesis focused on the impact of fluid restriction protocols, assessing to what extent the protocols physiologically and behaviourally affect rhesus macaques. However, in addition to exploring the impacts of scientific protocols, it is important, where possible, to refine their use. Indeed, this was a key point made by Prescott et al. (2010), who emphasised the need for researchers to choose their reward schedules and reward types carefully in order to optimally motivate macaques to work adequately under less restrictive regimes. A variety of motivational techniques are used in the literature and, to the best of my knowledge, there are currently no data that compare the effectiveness of different reward schedules.

There are three aspects of reward that seem likely to be effective at increasing motivation for animals to perform in tasks, allowing restriction protocols to be relaxed. The first is the use of preferred rewards. The expectancy of receiving a preferred reward is evident in increased activity at a neuronal level in macaques and is coded separately from the physical or taste properties of a reward (Cromwell et al., 2005; Tremblay and Schultz, 1999; Watanabe, 1996). In addition, damage to the cortical areas encoding the information results in impaired reward valuation (Baylis and Gaffan, 1991). Preference can also be demonstrated behaviourally, with macaques reaching more quickly for a favoured food reward over a less preferred reward (Watanabe et al., 2001) and performing longer anticipatory licks for preferred fluids (Hassani et al., 2001; Watanabe et al., 2001). Furthermore, preferred items function more effectively as rewards (Fisher et al., 1997; DeLeon et al., 2001) and can result in more successful training of behaviour (Clay et al., 2009).

The second potential motivator is variability, which can be introduced in two forms. Firstly, when researchers choose not to reward every correct trial or every n^{th} correct trial that a monkey performs, but instead introduce variability into the schedule by rewarding monkeys on a random or pseudorandom basis. The efficacy of using such

variable ratio (VR) schedules has been encouraged as a possible tool to refine or replace fluid restriction protocols (Westlund, 2012). Secondly, researchers can introduce variability or variety in the types of rewards they use. In early work following on from Skinner (1953), Wunderlich (1961) demonstrated that using varied rewards (food or fluid) helped to strengthen the resistance to extinction of task learning, compared to when using each reward alone or simultaneously. . Further work has also demonstrated that rats will perform at a better rate when their rewards are varied throughout a task (Melville et al., 1997; Bouton et al., 2014)

Finally, giving monkeys a choice of reward may also enhance motivation. At the neuronal level, it has been demonstrated that the act of choosing a reward, rather than simply receiving one, may have intrinsic motivational value, separate from the hedonic or nutritional value provided by the fluid reward (Tremblay and Schultz, 1999). Moreover, presenting both animals and humans with free choice is also known to be preferred over a forced choice alternative (Brigham and Sherman, 1973; Catania and Sagvolden, 1980; Fisher et al., 1997). Finally, in addition to the potential motivational and reinforcing value of choice, choice has also been advocated in the promotion of improved animal wellbeing (Catania and Sagvolden, 1980; Rumbaugh and Washburn, 2008)

4.2 Aims

Taking into consideration the importance of selecting rewards, I first aimed to assess the types of foods and fluids used to motivate macaques in scientific study by conducting a literature search. Given the potential efficacy of using preferred rewards and the possible motivational value of VR schedules and choice reward schedules, this chapter then had two main experimental aims. Firstly, I aimed to find a way to efficiently quantify fluid reward preference in rhesus macaques, and secondly, to use these preferences to compare the motivational capacities of different reward schedules: the monkeys' previous reward, their new preferred reward, a VR of previous and preferred rewards, and a choice of the previous and preferred rewards. I measured whether fluid preference could be established in the laboratory or home cage, whether the use of different reward schedules could maintain sufficient motivation and finally, if the level

of motivation was high enough to sustain adequate task performance when their fluid restriction was lessened.

4.3 Literature Search

4.3.1 Methods

I conducted a literature search to assess how rewards and restrictions are reported in studies utilising macaques. Three searches were carried out on The Web of Knowledge^{SM} database (v. 5.10) using the following combinations of keywords: 'macaque and neuroscience', 'macaque and learning and behaviour' and 'macaque and electrophysiology and behaviour' to examine papers from 2010 - 2016. Searches were refined by selecting for articles and by excluding reviews. Relevance of an abstract was assessed on the study being laboratory based (i.e. not a field study) with the use of a monkey and the possibility of a task being performed. Studies were excluded at this stage if they did not utilise a species of macaque, if the study was not carried out in a laboratory or home cage environment, or if the animal was not used in a rewarded protocol. Information was extracted from suitable papers for the following parameters: species used, reward type, reward amount, access to fluid and access to food.

4.3.2 Results

In total, 124 of the returned results were suitable for inclusion in the dataset (see full table in Appendix B). Initially, I had hoped to gain insight into the types of rewards given to macaques, but instead uncovered a lack of reporting within the literature (results are summarised in Table 6). Of 124 papers reviewed, 72 reported using some type of fluid reward, 41 used food rewards, 5 studies utilised both food and fluid rewards and the 6 remaining studies failed to specify what the monkey was rewarded with (Figure 8). However, the majority of studies reported only vague categories of food or fluid, such as "juice", "liquid", "fruit" or "pellet", leaving me unable to identify the specific rewards used.

Fluid Rewards

Exact rewards, including the type of fluid and the volumes given, were reported for 15 out of 77 studies using fluid motivators; the remaining 62 studies reported only

categories of reward. When detailing the provision of fluids (i.e. whether monkeys were given free access to water or whether restriction was required), 21 studies used some form of fluid restriction, with the remaining 56 studies failing to report specifics on the duration or frequency of fluid provision for the monkeys.

Food Rewards

Of the 44 studies using food rewards, 24 reported an exact reward and 20 gave a category of reward. When describing the monkeys' food provision schedules, 13 papers failed to state the frequency or duration of food access. There were some studies (6/44) which specifically made reference to food restriction and several (12/44) which explicitly stated that an animal was given *ad libitum* access or was not food restricted for the experiment. In contrast to evaluating fluid access, it was more difficult with the remaining 13 papers to determine whether food restriction was employed. Phrases such as, "they were maintained on a diet of fresh fruit, vegetables, and monkey chow" were unclear in conveying the amount of food being provided.

This analysis highlights a lack of reliable and informative reporting in the literature. The inconsistency of reporting as well as the variety of reward schedules employed are two of the reasons why monkeys' preferences need to be established. The results and implications of this literature search are discussed further in Section 4.6.1.

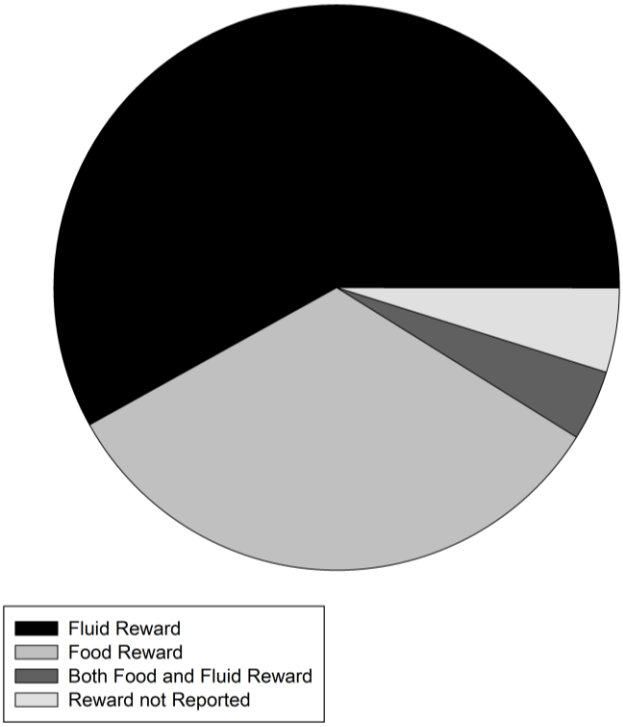


Figure 8. The reporting of rewards in 124 studies using macaques. 58% use fluid rewards, 33% use food rewards, 4% use both and 5% failed to report the reward used.

Table 6. The reporting of fluid and food provision in 77 studies using fluid rewards and 44 studies using food rewards.

Reward Type	Parameter	Level of Reporting	% of Papers Reporting
Fluid	Fluid Restriction	No restriction	0
		Restriction	27
		Not reported	73
		Ambiguous	0
	Reward type	Exact reward[a]	19
		Category[b]	81
Food	Food Restriction	No restriction	27
		Restriction	14
		Not reported	29.5
		Ambiguous	29.5
	Reward type	Exact reward[a]	56

	Category[c]	44

[a] An exact reward is defined as a reward that reports both a specific type of fluid or food as well as the amount given per presentation, using a description that would allow for replication of the reward schedule.

[b] Categories of fluids reported included: fluid (6%), liquid (25%), juice (44%), isotonic water with no defined volume (3%) and water with no defined volume (5%).

[c] Categories of foods reported included: food (9%), fruit (7%), pellet (14%), candy (7%), yoghurt (5%) and raisins and peanuts with no defined quantity (2%).

4.4 Experiment 1

4.4.1 Methods

Four monkeys were used in this study, weighing between 8 – 14.5 Kg at the start of the preference assessments. Minimum fluid intakes (Chapter 2, section 2.3) were as follows: Monkey 1: 250ml; Monkey 2: 200 ml; Monkey 3: 200 ml; Monkey 4: 385ml.

Establishing fluid preferences

Fluid preferences for each monkey were established by one of two methods. The first was to use the experimental set-up, where animals had already been trained to saccade to stimuli in order to access fluid rewards. A simple saccade choice task was devised, where looking at visually distinct stimuli presented on a screen resulted in different fluid rewards being delivered, allowing animals to choose which reward to receive. Fluid preference in the first two monkeys (Monkey 1 and Monkey 2) was assessed in this way. In each trial, they were initially required to fixate on a fixation spot (0.1 x 0.1 dva; 3 x 3 dva eye window allowance) for 3000 ms, after which three reward targets appeared. The monkey then had to saccade to any one of the three reward targets and fixate for 250 ms to complete a trial correctly and receive an associated fluid

reward (~0.1 – 0.2 ml fluid). Failure to fixate on a stimulus for long enough meant that the trial was terminated, and the animal was not rewarded. The three reward targets (each 2 dva; 3 x 3 dva eye window allowance) were equidistant from the fixation spot (0, 0) and were located at positions (x = -6.0, y = 6.0), (x = 0.0, y = -8.5) and (x = 6.0, y = 6.0). The location of the targets was randomised on each trial to control for any location bias and pseudo-randomisation was programmed such that targets occupied the locations for equal amounts of trials. Each reward target was distinguishable by colour for Monkey 1 (pink, red or blue) and by shape for Monkey 2 (annulus, triangle and diamond), each occupied the same area and was associated with a different fluid reward.

To investigate fluid preferences, the two monkeys had the choice between water (which they had previously received as a reward), and two fruit drinks, one nutritive and the other non-nutritive. The nutritive fruit drink was Ribena (40 ml of undiluted squash was added to 210 ml of water), which had been successfully used by other researchers in the primate facility to motivate their animals. Fruit tea (a cranberry and raspberry tea bag placed in 250 ml of hot water for 5 min, before being allowed to cool) had the taste of fruit without any high sugar content. The three fluids (water, Ribena and fruit tea) were delivered through a specially designed mouthpiece, which allowed three separate bottles to be connected via three plastic tubes so that there was no residue that could influence the taste of the next fluid. The bottles were calibrated prior to the experiments to ensure that the same amount of reward was delivered from each bottle. The fluid preference task was run for eight days and the fluid chosen on more than 50% of the days was taken as the monkey's preference. These preferences were then used to inform the design of the next part of the experiment which investigated the reward value of different motivational schedules.

The establishment of fluid preferences using the laboratory set-up was time-consuming, required additional apparatus (reward bottles and their associated control panels and a customised mouthpiece) and only allowed for three fluids to be tested. Using saccades to targets also has the potential to result in biases from the monkeys, for example, animals could always choose a particular type of stimulus or locations. Given that I wanted to find refinements to fluid restriction protocols that are easy to implement in a behavioural neuroscience setting, I decided to test fluid preferences for

Monkeys 3 and 4 using a second methodology in the home cage in an attempt to screen more fluids in a simpler and quicker task.

The home cage preference assessment was conducted with Monkeys 3 and 4. As they would not be "earning" their fluids, they could not be fluid restricted and so had free access to water during the days on which they were tested. Initial assessments consisted of a range of different juices (apple, pineapple, mixed fruits and orange) being presented in a choice paradigm to narrow down the options into two main preferences. The monkey was separated from his cage mate and juices were offered in syringes in pairs. Each juice was presented to the monkey to try before both juices were offered simultaneously. The juice preferred by the monkey (simply noted by which syringe he chose to drink from) was then refilled and presented alongside a new juice. The two most preferred fluids (chosen the most often) were then carried forward to the next experimental stage, along with the fluid with which the monkey had been previously rewarded in cognitive tasks. The fruit tea was not used here as it is not as viscous as the fruit juices and the bottles used in the cage, unlike the bottles in the experimental set up, cannot be calibrated to dispense equally. Therefore, by using juices of similar viscosity, I hoped to control for the amount that could be drank from the bottle in the next stage of the experiment.

The two most preferred fluids and the monkey's previous fluid reward were presented in 1L bottles attached to the cage in left, middle and right hand positions. Upon presentation, the monkey had 5 min of access to the bottles, after which time the volumes consumed were recorded. Five min allowed enough time for the monkey to drink, without potential post-ingestive effects biasing the data (Pritchard et al., 1994). The 5-min test was carried out at the same time each day (between 9:00 – 10:00) for six d. Each day, the bottles were spatially arranged in a unique way that allowed every combination of fluids and positions to be presented once, which controlled for place preferences. The fluid chosen consistently over the 6 d (on 50% or more of the days) was then used as the preferred reward when testing the reward schedules in the laboratory.

Assessing the motivational value of different fluid reward schedules

After fluid preference had been established by either method, each monkey performed a laboratory task with which they were familiar whilst fluid restricted at their normal level (as established and implemented in past studies and as calculated in Chapter 2, Section 2.3). Monkey 1 conducted a passive fixation task (ignoring a presented stimulus and keeping fixation on a central cue), Monkey 3 performed a bar release task (releasing a touch bar after a change in a stimulus cue) and Monkey 4 carried out a fixation task (fixating on a central cue). Although the tasks differed between monkeys, the nature of the task was irrelevant; it was only important that a monkey was familiar with a task and could consistently perform it in order to measure the effectiveness of the different reward schedules.

The monkeys performed their task and received one of four different reward schedules on different days. For the completion of a correct trial, the monkeys received either the reward given to them in previous studies (previous reward), their preferred reward as established in the preference assessment (preferred reward), a combination of the two (50% chance of receiving either previous or preferred: named the variable schedule) or were given a choice between previous and preferred rewards (choice schedule). In the choice schedule, the monkeys were required to choose between their previous reward and preferred reward by making a saccade upon completion of a correct trial. A cross-shaped stimulus represented the previous reward and a circle represented their preferred reward. The stimuli were presented at (x = -6.0, y = 0) or (x = 6, y = 0) dva and the monkeys were required to fixate for 250 ms to gain the reward. The reward schedules were carried out in four blocks of four days, with schedules randomised within blocks. The number of correct trials performed was recorded on each day.

To assess whether the monkeys' performances on their respective tasks could be maintained under less restrictive fluid restriction conditions, the daily fluid intake of the monkeys was increased by 100 ml. Raising the fluid allowances allowed me to assess whether any of the reward schedules would be effective at a less restrictive fluid restriction protocol, i.e. equal (or similar) performance in terms of trial numbers.

4.4.2 Statistical Methods

All data were checked for normality and equal variances, and analysed using appropriate parametric or non-parametric tests in IBM Corp. SPSS (v21, SPSS Inc, Chicago, USA). Fluid preferences were tested using one-way ANOVAs or Kruskal Wallis tests. The effectiveness of the reward schedules was tested using Kruskal Wallis tests, mixed models and *t* tests. Finally, I compared the performance levels to 1000 trials (an adequate performance in the laboratory) using one-sample *t* tests for each monkey, with a test value of 1000.

All pairwise tests were corrected for multiple comparisons using false discovery rate (FDR) *post-hoc* tests (Benjamini and Hochberg, 1995). To do this, the *p*-values were taken from the pairwise comparisons and ranked from lowest to highest. The standard alpha significance value of 0.05 was then divided by the number of comparisons made and all numbers below this to 1. It was then determined whether the smallest *p*-value was smaller than the corrected alpha level (*p*/number of comparisons, which is termed the *q*-value in FDR statistics). If so, the correction factor (number of comparisons) was adjusted, whereby it was reduced by 1, yielding a new accepted alpha level (*q*-value). I then determined whether the second smallest p-value was smaller than the new alpha level (*q*-value). If so the procedure was repeated until the respective ordered *p*-value exceeded the currently relevant *q*-value. The adjustments were stopped whenever the ranked *p*-value exceeded the corresponding *q*-value.

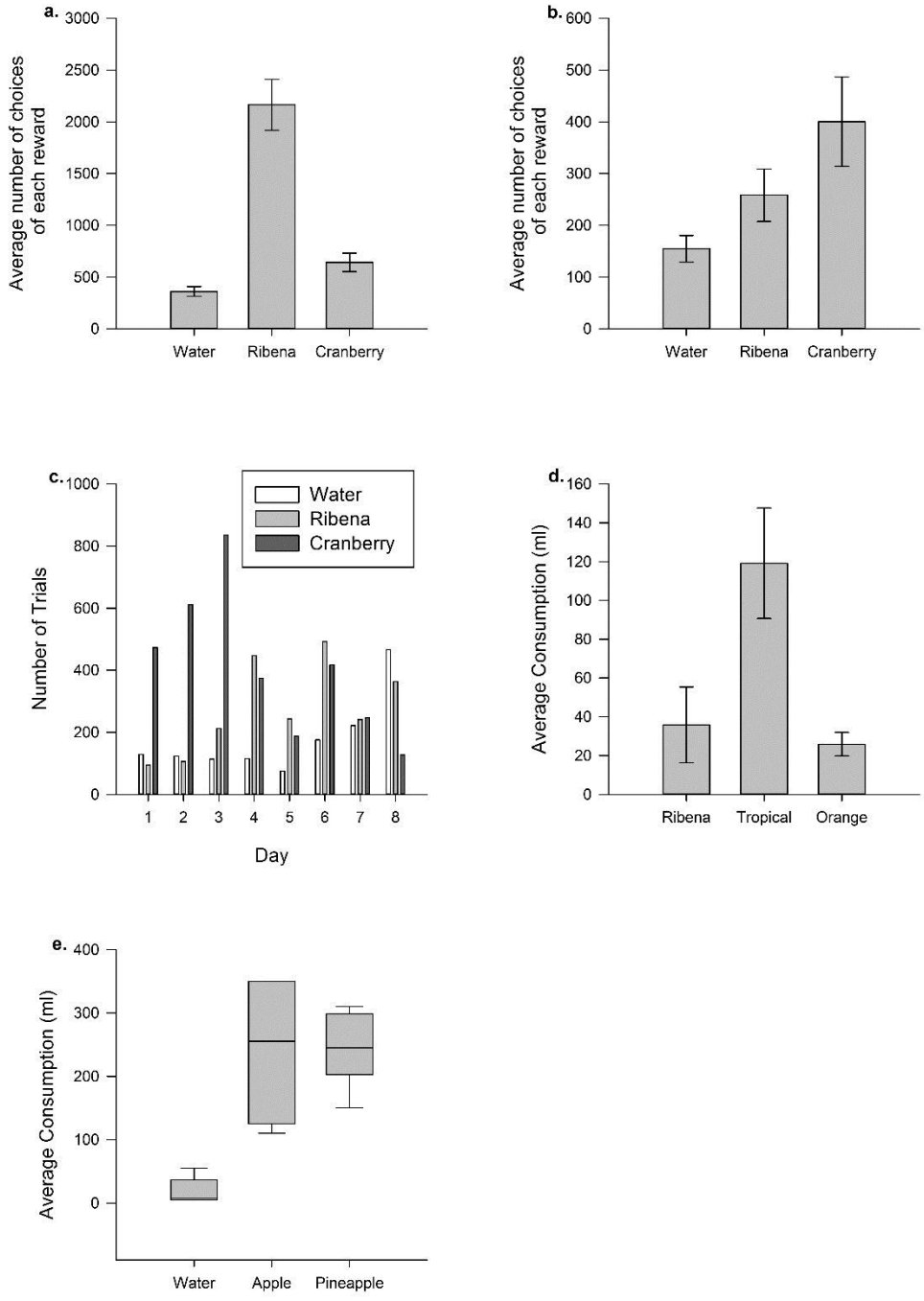
4.4.3 Results

Establishing fluid preferences

To establish fluid preferences from the three fluids given to each monkey, either a one-way ANOVA (Monkeys 1, 2 and 4) or Kruskal Wallis test (Monkey 3) was used, to compare the number of choices for each fluid in the laboratory (Monkeys 1 and 2), or the amount of each fluid consumed in the home cage (Monkeys 3 and 4). When using the laboratory set-up, a clear fluid preference could only be established for Monkey 1. Monkey 1 differentially chose the three different fluids (ANOVA, $F_{(2,15)} = 48.62$, $p < 0.001$; Figure 9), preferring Ribena to both cranberry tea ($t_{(10)} = 6.78$, $q < 0.05$) and water ($t_{(10)} = 9.64$, $q < 0.05$) and preferring cranberry over water ($t_{(10)} = 3$, $q < 0.05$). Whilst Monkey

2 also showed a significant difference in the amount of each fluid he chose (ANOVA, $F_{(2,21)} = 3.89$, $p = 0.037$; Figure 9b), this was not consistent across days (occurred < 50% of test days) and was biased by a high intake of cranberry tea in the first three days of testing (Figure 9c). Due to this lack of consistent preference, Monkey 2 was not continued in the experiment, and did not experience the different reward schedules.

By using the cage method, fluid preferences were established for both Monkey 3 (Kruskal-Wallis, $H_2 = 11.43$, $p = 0.003$; Figure 9d) and Monkey 4 (ANOVA, $F_{(2,15)} = 5.83$, $p = 0.013$; Figure 9e). Monkey 3 preferred tropical juice to both his previous reward of Ribena ($t_{(10)} = 2.89$, $q < 0.033$) and to orange juice ($t_{(10)} = 3.42$, $q < 0.033$), with no difference between the orange juice and Ribena ($t_{(10)} = 0.091$, $q > 0.033$). Monkey 4 preferred both new juices over his previous reward of water (Apple: $U = 2.93$ $q < 0.033$; Pineapple: $U = 2.93$, $q < 0.033$) with no difference between apple and pineapple juice ($U = 0$, $q > 0.033$). Apple juice was chosen to be carried forward as his preference as there was a slightly more pronounced choice of this on the tested days (Median consumption: Apple 255ml, Pineapple 245ml).



Monkeys 3 and 4 in the home cage. The overall average number of choices (±SEM) made for the three possible rewards in the preference test for (a) Monkey 1 and (b) Monkey 2. Monkey 2 was not continued in the experiment as his fluid preference was not stable across the 8 testing days (c). The average consumption of the fluid rewards in 5 min over 6 days for (d) Monkey 3 and (e) Monkey 4.

Assessing the motivational value of different fluid reward schedules

Data from the reward schedule trials were not normally distributed and were analysed in multiple ways. Motivation was assessed individually at each fluid restriction level, by applying a Kruskal Wallis test with a fixed factor of reward schedule and a dependent variable of the number of trials completed. Monkey 1 received Ribena as his new preferred reward alongside water, which he had previously been rewarded with. At normal fluid intake levels, Monkey 1's performance varied across the four reward schedules (Kruskal Wallis, $H_3 = 12.40$, $p = 0.006$; Figure 10a). His highest performances were for Ribena or a variable reward schedule, which he performed equally well for (Mann Whitney, $U = 0.15$, $q > 0.017$). His motivation was lower for water compared to both of these schedules (Ribena: $U = 2.82$, $q < 0.017$; Variable: $U = 2.97$, $q < 0.017$). Although there was a trend suggestive of a decrease in performance when he was given a choice of reward, the number of trials was not significantly different from Ribena ($U = 1.78$, $q > 0.017$), the variable schedule ($U = 1.93$, $q > 0.017$) or water ($U = 0.30$, $q > 0.017$). Unlike Monkey 1, Monkeys 3 and 4 did not differ in their task performance for different fluid reward schedules (Monkey 3: Kruskal Wallis, $H_3 = 7.22$; Monkey 4: ANOVA, $F_{(3,12)} = 1.61$; $p > 0.05$ for both; Figure 10b and c), demonstrating that, for these two monkeys, the schedules had equal motivational value at a normal restriction level.

Approximately 1000 trials is considered an adequate level of task performance for behavioural neuroscience conducted in the laboratory. When rewarded with water, Monkey 1's performance did not differ from 1000 trials (one sample *t* test, test value = 1000; $t_{(3)} = 0.57$, $q > 0.0375$), and all other reward schedules elicited performance of over 1000 trials ($t_{(3)} < 6.89$, $q < 0.0375$ for all); showing all reward schedules to be adequately, or more than adequately, motivating. Taken together, these results suggests that the inclusion of Ribena within a schedule increased motivation to beyond that of water. Monkey 3's performance was no different from 1000 trials when rewarded with his previous reward (Ribena), preferred reward (tropical juice) or a variable schedule ($t_{(3)} < 2.41$, $q > 0.0125$ for all) but performance dropped lower than 1000 when he was given a choice of reward ($t_{(3)} = 19.84$ $q < 0.0125$). Monkey 4's performance did not differ from 1000 trials for any schedule ($t_{(3)} < 2.49$, $q > 0.0125$ for all), suggesting that all schedules were adequately motivating at the normal fluid restriction level.

The monkeys' fluid intake was increased by 100 ml for the next stage of the experiment to assess whether the reward schedules remained motivating when the monkeys were less restricted. At this relaxed fluid restriction, Monkey 1 and Monkey 3 performed different numbers of trials on the different reward schedules (Monkey 1: $H_3 = 8.70$, $p = 0.034$; Monkey 3, $F_{(3,12)} = 3.72$, $p = 0.042$; Figure 10a and b). However, Monkey 4 continued to perform a similar number of trials for each reward schedule (ANOVA, $F_{(3,12)} = 0.17$, $p > 0.05$; Figure 10c). For Monkey 1, the use of water alone produced similar performance to the variable schedule ($U = 1.93$, $q > 0.0083$) and the choice schedule ($U = 2.08$, $q > 0.0083$) but water resulted in a lower level of work than that achieved with Ribena ($U = 2.82$, $q < 0.0083$). There was no difference in performance between the variable, choice and Ribena schedules (Table 7). Monkey 3's performance when given a choice of reward was lower than when he was rewarded either with tropical juice ($t_{(6)} = 4.28$, $q < 0.017$) or variably rewarded ($t_{(6)} = 7.53$, $q < 0.017$), but there was no difference between any of the other reward schedules (Table 8).

Table 7. Monkey 1. Pairwise comparisons between the numbers of trials performed when rewarded with the previous or preferred rewards, the variable schedule and the choice schedule when the daily fluid allowance had been increased by 100 ml. The results are controlled for multiple comparisons using False Discovery Rate (FDR) tests. "NS" indicates a non-significant result.

Reward Schedule	Reward Schedule	Median Difference	*U*-value	Original *p*-value	FDR corrected *q*-value	Significance ($p < q$)
Previous	Preferred	1076	2.82	0.005	0.0083	Significant
	Variable	660	1.93	0.054	0.0083	NS
	Choice	864	2.08	0.038	0.0083	NS
Preferred	Variable	416	0.89	0.37	0.0083	NS
	Choice	212	0.74	0.46	0.0083	NS
Variable	Choice	204	0.15	0.88	0.0083	NS

Table 8. Monkey 3. Pairwise comparisons between the numbers of trials performed when rewarded with the previous or preferred rewards, the variable schedule and the choice schedule when the daily fluid allowance had been increased by 100 ml. The results are controlled for multiple comparisons using False Discovery Rate (FDR) tests. "NS" indicates a non-significant result.

Reward Schedule	Reward Schedule	Mean Difference	Std Error of Difference	*t*-value	df	Original *p*-value	FDR corrected *q*-value	Significance ($p < q$)
Previous	Preferred	83.25	236.79	0.35	6	0.74	0.017	NS
	Variable	61.25	215.30	0.28	6	0.79	0.017	NS
	Choice	419.75	211.61	1.98	6	0.095	0.017	NS
Preferred	Variable	22	123.98	0.18	6	0.87	0.017	NS
	Choice	503	117.46	4.28	6	0.005	0.017	Significant
Variable	Choice	481	63.88	7.53	6	<0.001	0.017	Significant

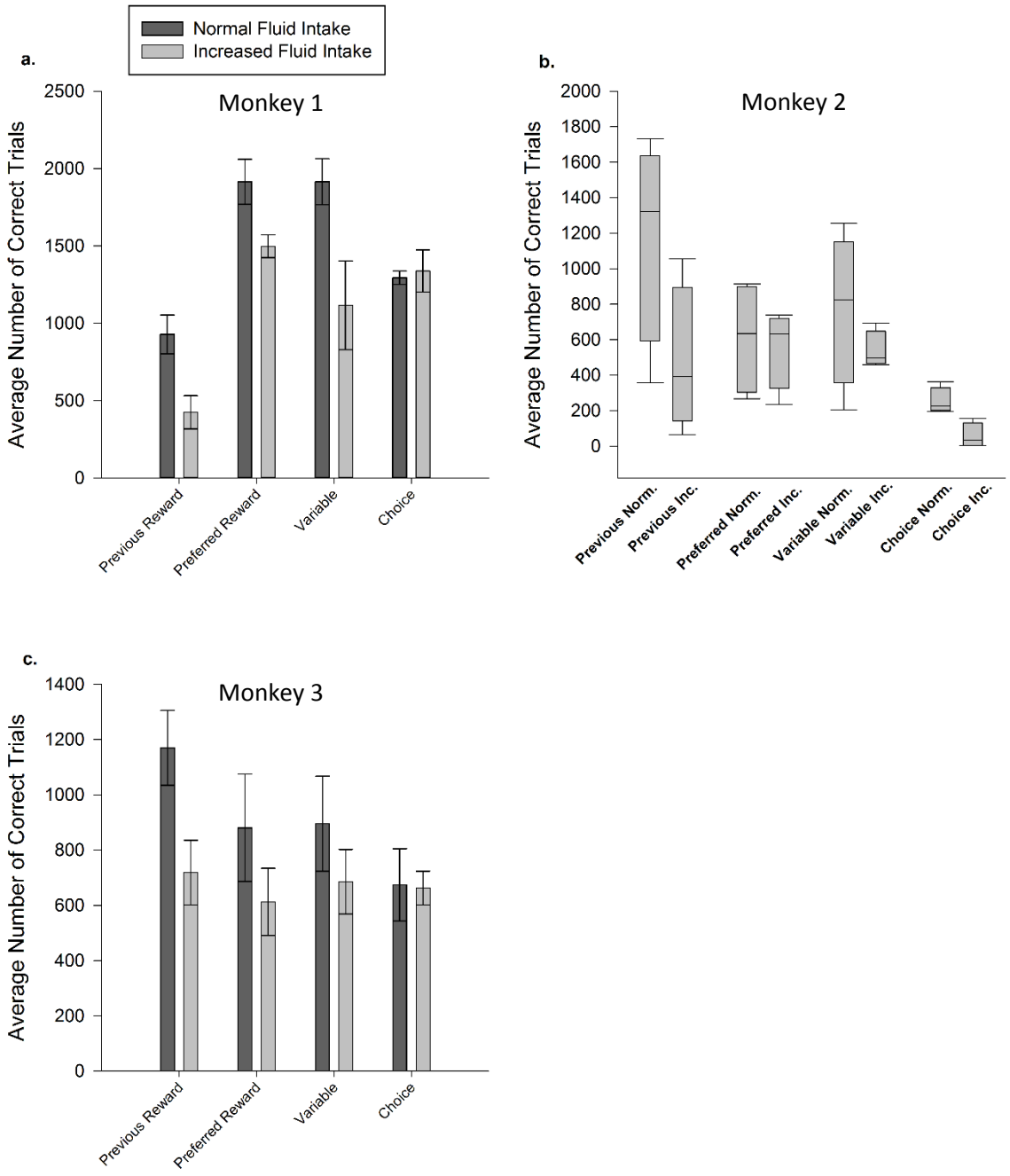


Figure 10. The average number of correct trials (±SEM) performed by (a) Monkey 1; (b) Monkey 3 and (c) Monkey 4 when rewarded with their previous reward, preferred reward, a variable schedule or a choice schedule at both their normal and increased fluid intakes. In (b) "Norm." refers to the normal fluid intake and "Inc." to an increased fluid intake.

Again, I compared the monkeys' performances to 1000 trials to ascertain if any schedule resulted in an adequate laboratory performance. Monkey 1's daily performance remained around 1000 trials when rewarded with the choice and the variable schedules ($t_3 < 2.47$, $q > 0.025$), and he performed over 1000 trials when rewarded with Ribena ($t_3 = 6.67$, $q < 0.025$). However his performance was below adequate (<1000) when rewarded with just water ($t_3 = 5.36$, $q < 0.025$); suggesting that his previous reward had now decreased in motivational value whilst the preferred reward continued to be motivating. In contrast, Monkey 3's performance with his previous reward of Ribena remained at around 1000 trials ($t_3 = 2.51$, $q > 0.0375$) whereas the trials dropped below 1000 when he was rewarded with his preferred reward, the variable schedule or with a choice of rewards ($t_3 < 26.67$, $q < 0.0375$ for all), indicating that Ribena was the only motivating fluid at this restriction level. For Monkey 4, trials completed for his previous reward, preferred reward and variable schedule did not differ from 1000 ($t_3 < 3.19$, $q > 0.0125$ for all) but did fall below 1000 for the choice schedule ($t_3 = 5.52$, $q < 0.0125$), demonstrating the lack of value this had as a reward schedule at an increased fluid intake.

When assessing all elements of the study as a whole, I looked for overall effects as well as an interaction between the reward schedule and fluid restriction level using a fully factorial ANOVA with reward schedule and fluid restriction level as fixed factors. Monkey 1 and Monkey 3's performances showed an overall effect of fluid intake level (Monkey 1: $F_{(1,24)} = 15.70$, $p = 0.001$; Monkey 3: $F_{(1,24)} = 7.80$, $p = 0.01$) and reward schedule (Monkey 1: $F_{(3,24)} = 17.93$, $p < 0.001$; Monkey 3: $F_{(3,24)} = 8.50$, $p = 0.001$), but no interaction between the two (Monkey 1: $F_{(3,24)} = 2.73$, $p > 0.05$; Monkey 3: ($F_{(3,24)} = 1.31$, $p > 0.05$). However, Monkey 4 did not show any significant differences for fluid intake level ($F_{(1,24)} = 4.23$, $p > 0.05$), reward schedule ($F_{(3,24)} = 1.14$, $p > 0.05$) or for the interaction of the two ($F_{(3,24)} = 0.74$, $p > 0.05$).

Finally, I assessed changes in performance for each of the different reward schedules from when fluid restriction was changed from the normal level to the increased level, and carried out *t* tests for each schedule to establish any change in the number of trials performed. Monkeys 1 and 4 showed no differences between their performances at the different fluid intakes for any of the reward schedules. Monkey 3, however, had a significant decrease in trials performed in the choice reward schedule when daily fluid

intake was increased (Mean Difference = 196.25, $t_{(6)} = 3.80$, $q < 0.0125$), but no change for any other schedule (Table 9).

Table 9. The difference in performance for each monkey at each reward schedule when the fluid allowance was increased. The results are controlled for multiple comparisons used False Discovery Rate (FDR) tests. "NS" indicates a non-significant result.

	Reward Schedule	Mean Difference	Std Error of Difference	*t*-value	df	Original *p*-value	FDR corrected *q*-value	Significance after FDR corrections
Monkey 1	Old	503.25	164.79	3.054	6	0.022	0.0125	NS
	New	416.75	163.48	2.549	4.48	0.057	0.0125	NS
	Variable	799	322.67	2.476	4.51	0.062	0.0125	NS
	Choice	44.25	143.41	0.309	6	0.77	0.0125	NS
Monkey 3	Old	708.5	359.18	1.97	6	0.096	0.0125	NS
	New	53.5	196.08	0.273	6	0.79	0.0125	NS
	Variable	240.50	222.33	1.082	6	0.32	0.0125	NS
	Choice	196.25	51.70	3.796	6	0.009	0.0125	Significant
Monkey 4	Old	451.25	179.28	2.517	6	0.045	0.0125	NS
	New	268	229.30	1.169	6	0.29	0.0125	NS
	Variable	210.25	207.81	1.012	6	0.35	0.0125	NS
	Choice	11.75	144.40	0.081	6	0.94	0.0125	NS

4.5 Experiment 2

Within the primate facility some of the female macaques are exposed to an automated testing system at their home cage. This device is part of a separate project, investigating whether training in the home cage can translate into improved performance of a motor task in the laboratory. The monkeys are cued to press specific buttons to receive a fluid reward. In collaboration with this project, I tested the fluid preferences of each of the females using the system and the researcher heading the study then used these preferred fluids to assess whether performance could be enhanced on the automated system.

4.5.1 Methods

Six female monkeys aged between 3 and 6 years old and weighing between 4.8 – 6 Kg were tested. All were pair-housed with another female, but testing with the automated system was carried out when the animals were separated from one another. The amount of training sessions differed for each monkey depending on husbandry procedures and laboratory schedules. For full details of the automated training system see Tulip (2015).

Fluid preference testing had to be brief and fairly informal so as not to encroach on the studies for which these macaques were primarily being used. As previously described in this section, juices were presented to the monkeys in syringes in their home cages and the preferred from each pair was refilled and presented with a new juice. Using this method, a preference was established for each monkey. Although not as stringent as other preference tests, this method allowed me to quickly assess a juice preference without potentially impairing the monkeys' laboratory training sessions. Each monkey's performance was recorded for three weeks on their previous reward (Ribena in all cases) before the new preferences were then implemented. New preferences were given for between 2 – 11 sessions, dependent on the monkey, and the number of correct trials performed was recorded for each session.

4.5.2 Results

For the females using the automated system, the data were not normally distributed, and were transformed to normality using a square root transformation. A

mixed model was used to assess whether the change in reward fluid had impacted on the number of trials performed by the monkeys. The reward fluid (previous or preferred) was used as a fixed factor and a random effect of monkey was added. Changing the reward fluid in the automated reward system did not have an effect on the average number of trials performed by the monkeys ($F_{(5,93.12)} = 0.86$, $p = 0.36$; Figure 11), indicating that the new, preferred rewards were no more motivating than the previous reward.

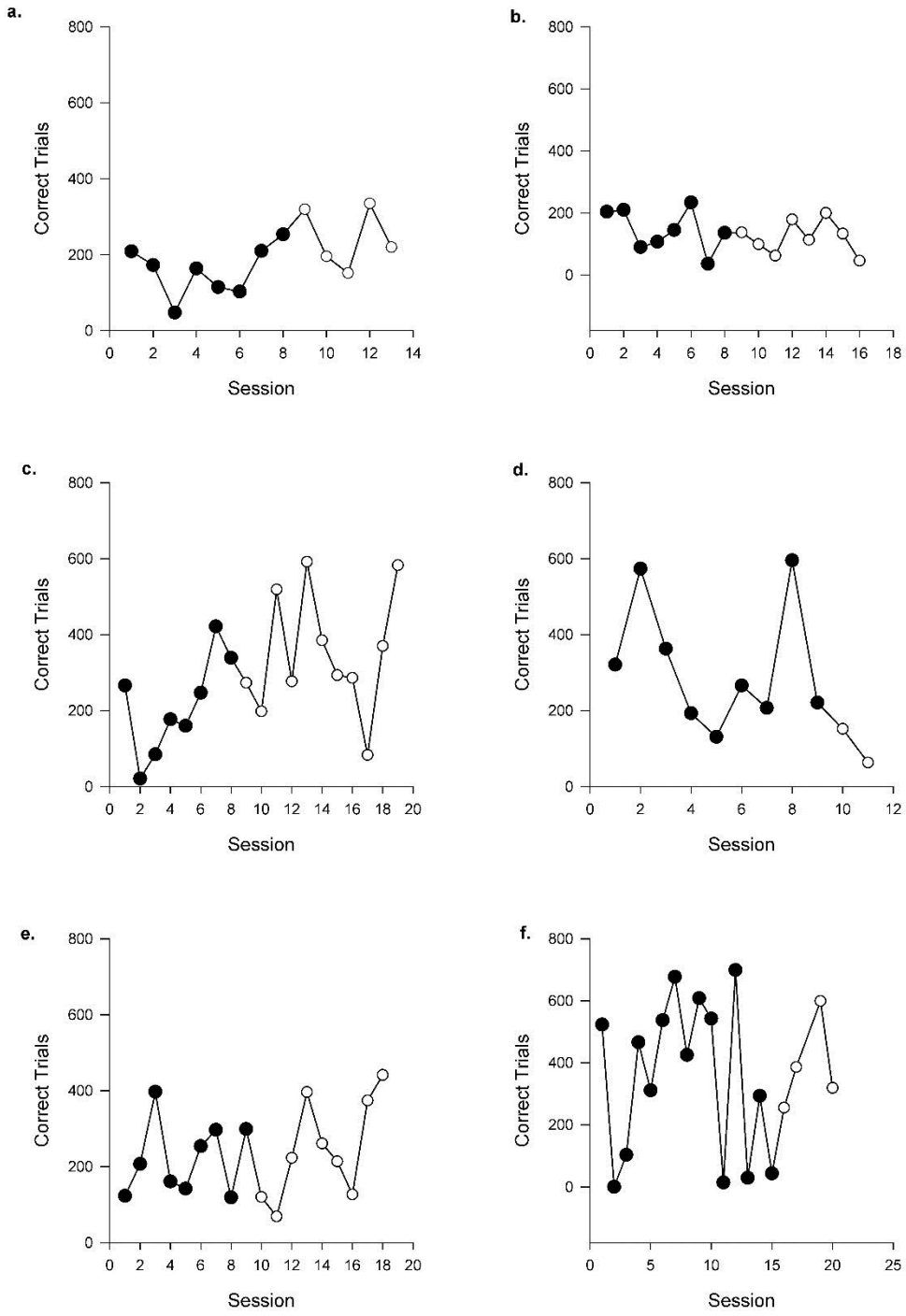


Figure 11. The number of correct trials performed by six female macaques using an automated training system. The monkeys are cued to press certain buttons to gain fluid rewards. Filled circles represent reinforcement with the previous reward (Ribena in all cases) and open circles represent training sessions using their new, preferred rewards

4.6 Discussion

This study investigated the effects of using preferred fluids in different reward schedules and the resulting impact on macaque motivation, as well as the potential to use the schedules in refining current fluid restriction practices. In addition, I explored the practicality of assessing fluid preference in rhesus macaques in both the experimental set-up in the laboratory and using bottles placed in the home cage. There were advantages and disadvantages to both methodologies and the potential explanations and modifications are discussed below.

4.6.1 Literature Search

The first aim of this study was to explore the types of foods and fluids used to reward macaques in scientific study. However, instead of fulfilling this aim, the analysis of 124 studies inadvertently uncovered a lack of reporting of reward and restriction in the literature. To fully appreciate how results are gained in a study and to be able to replicate methodologies, there needs to be clear reporting. The type of reward given, the amount of reward provided per correct trial and whether the monkeys needed to be in any way restricted to be able to perform the task (either by time of access to food/fluid or by amount of food/fluid), should be clearly stated in a publication. The scientific community working with primates is fairly small and techniques and motivational methods should be easily accessible in the literature in order to allow for successful protocols to be adopted by others.

4.6.2 Preference Testing

Initially establishing fluid preferences in macaques is important in order to be able to attempt refinement to commonly used fluid restriction protocols (Prescott et al., 2010). It was hoped that by identifying preferences, adequate levels of task performance could be maintained from the monkeys under less restrictive conditions. However, I failed to establish a stable preference for one of the monkeys in this study, as his choice for different fluids fluctuated throughout testing. For the remaining three monkeys, preferences were successfully established, though these were not always translatable into effective rewards (see Section 4.6.3).

I attempted to establish fluid reward preference using two methodologies; assessments made in the laboratory and in the home cage. As previously discussed, assessments made using the laboratory set-up brought with it related issues, such as potential biases, the need for additional equipment and longer training times. In contrast, assessing fluid preference in the home cage was much quicker and simpler; the protocol took five min a day for six days with only 3 bottles needed as equipment. However, this method was not without its drawbacks, with the main being that fluid restriction was not in place as restriction is not permitted unless the monkey has the opportunity to 'earn' as much fluid as he likes. The home cage assessment did not meet these requirements as the monkeys did not have to work for the juices and were only given five min access a day to the fluids of interest. This meant that the testing had to be conducted whilst the monkeys had free access to water in their home cages which may have confounded their fluid choices.

4.6.3 Reward Schedule Testing

The monkeys' individual fluid preferences were used in different reward schedules to assess their effectiveness as motivators. The preferred rewards and previous rewards were presented alone, as well as in a variable schedule (with 50% chance of receiving either) and finally, a choice between the two. Importantly, it was uncovered that each monkey responded differently to the reward schedules and that there were no uniform results, highlighting the significance of treating macaques as individuals when assessing effective rewards.

All four schedules were rewarding when the monkeys were subject to their normal level of fluid restriction when they could be reliably assumed to be motivated to drink. Performance rates were adequate or above adequate for all schedules, with the exception of the choice schedule for Monkey 3 (in which performance dropped below 1000 trials). I expected that the inclusion of a preferred reward in a schedule would increase the monkeys' motivation, which was the case for Monkey 1; his best performances were when rewarded with his preferred reward or a variable schedule. Surprisingly, though, this pattern was not seen in Monkeys 3 and 4, which could be a result of assessing their reward preference when they were not fluid restricted. Being satiated by water before and after preference assessments may have confounded their

fluid choices, rendering these preferences less effective as rewards when the monkeys were fluid restricted later in the study. In addition, the female monkeys using the automated reward system were also no more motivated by their new preferred reward when compared with their previous juice reward of Ribena. The females were not fluid restricted at any point during the assessments and their performances were very variable from session to session, whether rewarded with previous or preferred rewards. The relatively low numbers of sessions and trials per day for the females may have meant that changes in motivation might not have been detectable, or it may be that the lack of increase in motivation was a true result; it is not possible from the data to disentangle this.

To assess whether the reward schedules continued to elicit high levels of motivation during a more relaxed fluid restriction protocol, the monkeys' daily fluid intake was increased by 100 ml. Again, it was expected that the preferred fluid, the variable schedule and the choice schedule would be motivating to the monkeys. In line with these predictions, I found poorer levels of work for the previous reward of water for Monkey 1 and, to a certain extent, Monkey 4. In contrast, Monkey 3 continued to perform adequately (approximately 1000 trials) when receiving his previous reward. This is perhaps because Monkeys 1 and 4 had previously been rewarded with water, and Monkey 3 with Ribena. The monkeys are supplemented with water if they have not reached their daily intake allowance via task performance and thus for Monkey 3, it may be that Ribena remained motivating when he had learned he would receive only water afterwards. Conversely, for Monkeys 1 and 3, it was probably less motivating to be rewarded with water, as it could be received for "free" after work, especially when their motivation to drink was lower and they could afford to wait for their water.

Unlike the preferred reward alone, the choice protocol and variable protocol yielded lower performances than anticipated, bringing into question their efficacy as motivators. For the choice schedule, this was likely due to the additional effort that was required. The monkeys first had to perform the initial task correctly, before being offered choice, i.e. a trial took longer, and it required additional cognitive operations. While choice is often seen as potentially rewarding (Brigham and Sherman, 1973; Catania and Sagvolden, 1980; Fisher et al., 1997), these findings suggest that the costs

and benefits in a laboratory setting can balance out. Although the variable schedule produced some promising results at the normal fluid intake level, performance dropped for two of the monkeys when the fluid restriction was relaxed. The inconsistency seen in performance when the fluid intake was increased could have been a result of the animal being less motivated to drink. On some days receiving a preferred juice intermittently may have been motivating enough to continue working, whereas on other days, especially for those monkeys receiving water as their previous reward, this may not have been adequately motivating to keep performance levels high. Variability in reward size, and the consequential unpredictability, has been demonstrated to result in dopamine release in macaques, particularly when the chance of receiving a reward is at 50% (Fiorillo et al., 2003). It is maybe a little surprising then, that variability in the reward fluid received at a level of 50% is not more motivating at the increased fluid intake. However, some evidence does suggest that variable schedules are less motivating to primates than they are to other species (Bowman *et al*., 1996).

4.7 Conclusions

This chapter demonstrates that obtaining a fluid preference improved performance in a cognitive task in some monkey, even when fluid restriction was relaxed, although there was variation in preference and performance between monkeys. Interestingly, choice and variable schedules were not as rewarding as I had anticipated. It is important to note that each monkey had an individual response to both the preference testing and the reward schedules and that 'blanket' protocols should not be applied to all macaques. I also highlight the potential benefits and flaws of fluid preference testing for rhesus macaques. From these findings, I believe that fluid preference should ideally be assessed when the animal is in the home cage but fluid restricted. This would allow for quick and simple testing whilst keeping the monkey's motivation to drink equal to that experienced when performing a task in the laboratory, thereby reducing the confounding factor of water satiation.

Chapter 5: The Efficacy of Social Stimuli as a Refinement to Fluid Restriction Protocols

5.1 Introduction

So far, my thesis has focussed on the impact of fluid rewards on macaques (Chapter 3) and the potential refinements to their application in experimental procedures (Chapter 4). However, there is a need to explore alternative options to fluid reinforcement. One of the possibilities suggested by Prescott et al. (2010) is the use of social stimuli, using images or videos of conspecifics as a means of reward.

There is reason to believe that macaques could be motivated by social rewards, as they live in groups and form strong social bonds. In the wild, macaques live in large mixed-sex and mixed-age groups of varying sizes, dependent on habitat type and resource availability (Seth and Seth, 1986; Lu et al., 2007). Macaques engage together in play, social contact and foraging (Southwick et al., 1965; Lindburg, 1977) and a large proportion of their time is dedicated to social activities, which remain integral to their daily routines even throughout the summer when more time is required to locate food and water (Malik, 1986).

Although rhesus macaques are a highly social species and adapted to group living, extensive grouping is not always possible in UK scientific holding facilities, and more typically, animals are housed in smaller social groups or in pairs. In exceptional circumstances, such as when a monkey is in a pre- or post-operative state or following bouts of aggression with a cage mate, animals may have to be housed alone. Given that in these holding conditions animals have less social contact than their conspecifics in the wild, it is worth speculating that macaques' natural sociality could be capitalised upon when exploring alternative rewards. Indeed, some early studies of social reward demonstrated that rhesus macaques would open a window in order to gain visual access to a conspecific (Butler 1954). Since then, numerous studies have shown that NHPs will perform a wide range of tasks to gain social rewards, including: lever pressing (Sackett, 1965; Swartz and Rosenblum, 1980), pushing on a panel (Fujita and Matsuzawa, 1986) and manipulating a joystick (Andrews et al., 1995; for full review see Anderson, 1998).

In addition to this, preferences for viewing specific types of social rewards have also been reported. For example, both male and female rhesus macaques prefer viewing photographs of unfamiliar, rather than familiar, conspecifics (Haude and Detwiler, 1976; Platt and Novak, 1997); female stump-tailed macaques (*Macaca arctoides*) show more interest in photographs of infant stump-tailed macaques and females carrying infants compared with pictures of lone adult females (Demaria and Thierry, 1988); and female rhesus macaques choose to view faces of dominant male macaques but are less interested by low-status males (Watson et al., 2012). This suggests that the social structure of macaques determines specific preferences in social stimuli and that images are viewed to gain social information.

However, whilst these kinds of social stimuli are clearly of interest to macaques, the question remains as to whether they are sufficiently motivating to function as a reward for adequate task performance. Some studies suggest that this may be the case, and argue that social stimuli provide a viable alternative or supplement to fluid or food rewards. In a task where male rhesus macaques chose between a smaller fluid reward coupled with a social image or a larger fluid reward with no image, males would sacrifice fluid to view images of dominant males and female perinea, but required an 'overpayment' of fluid to view subordinate males (Deaner et al., 2005; Klein et al., 2008). Other studies suggest that interest in viewing videos and photographs can be maintained by changing stimuli sets (Platt and Novak, 1997; Andrews and Rosenblum, 2001; Ogura and Matsuzawa, 2012), indicating that novelty may be an important factor in prolonging interest. Although these examples demonstrate that social stimuli can be rewarding, some of the studies (Andrews and Rosenblum, 2001; Ogura, 2011; Ogura and Matsuzawa, 2012) were carried out with macaques that were individually housed, a factor which could increase the reward value of the stimuli.

Taking into account the evidence surrounding the potential motivational value of social stimuli, this chapter aims to test to what extent social stimuli can be used to motivate macaques in a behavioural neuroscience setting for adequate task performance, with the aims to reduce the need for fluid rewards, and refine protocols associated with fluid restriction. Both greyscale and colour images were assessed to explore whether they had different motivational values, as well as greyscale video clips.

5.2 Methods

Four monkeys were used in this study, weighing between 8 – 14.5 Kg at the start of the preference assessments. Monkey 1 and Monkey 2 were shown greyscale images and videos, whereas Monkeys 3 and 4 were used in evaluating colour images. Minimum fluid intakes (Chapter 2, section 2.3) were as follows: Monkey 1: 200 ml; Monkey 2: 250 ml; Monkey 3: 415 ml; Monkey 4: 190 ml. Monkeys 1 and 2 were dominant and Monkeys 3 and 4 were subordinate.

5.2.1 Stimuli Collection and Editing

Photographs and videos (3072 x 4608 pixels, 24 bit colour depth) of adult rhesus macaques were collected from the German Primate Centre (Deutsches Primatenzentrum, DPZ) in Göttingen, Germany, in October 2013 using a Nikon 1 V2 camera. Photographs were taken of macaques in large, outdoor enclosures, in which they were socially housed. Of the images taken, a subset of neutral faces from dominant males (either face forward view or profiled view) and of female perinea were selected to be edited in both greyscale and colour to produce the below image sets. The backgrounds of the photographs were removed to avoid any elements of interest detracting from the focal image. Backgrounds could not be removed from the videos. Due to the difficulty in obtaining videos of lone adult females at the DPZ (females were mostly situated in groups with their infants), further videos were recorded using female rhesus macaques in the Comparative Biology Centre, Newcastle (the facility in which the males in this study were housed) in March 2014.

5.2.2 Greyscale Stimuli

70 greyscale images were used in total (35 male and 35 female). Seven males were photographed, with 5 images used from each individual. Due to the difficulty in obtaining good quality images of female perinea, seven females were photographed, with 2-7 usable images for each individual.

The original photographs were converted to greyscale and were edited to normalise contrast and luminance across all photographs. Control images were produced by scrambling the facial and perineal images such that the distributions of contrast,

luminance and spatial frequency were identical to the original image (Figure 12a). This was achieved by performing a Fourier transformation and calculating the amplitude and phase spectrum of this transform. A random phase structure was then generated and added to the phase spectrum of the Fourier transform. The amplitude spectrum was then combined with the phase spectrum and an inverse Fourier transformation was performed. Scrambled images were chosen over other types of control images, such as landscapes, because they retained the second order statistics of the original images, allowing me to test the efficacy of the social stimuli, without the complication of containing other potentially motivating/rewarding features in the control image. The control images were therefore sufficiently neutral to test whether social rewards can be used as supplements or replacements of fluid control, whilst ensuring that other differences (e.g. second order image statistics) between social and control stimuli did not confound the interpretation of the results. All social images and control images were resized to 326 x 326 pixels.

Greyscale videos (28 male videos and 28 female videos) were created by editing videos into 2000 ms lengths and then splitting these clips into their component individual frames. Individual frames could then be treated as photographs and converted into greyscale and normalised for contrast and luminance as above. The frames were then spliced back together into a video format suitable for presentation in the laboratory (.ctx file extension). Controls were not created for the video stimuli due to experimental design (see section 5.2.6).

5.2.3 Colour Images

The same set of photographs used for the greyscale images was used for testing colour images. However, the contrast in one male's images made it impossible to enable colour normalisation, and this individual was omitted from the colour image set, leaving 30 male images. Five images (the poorest in quality, e.g. more blurred) were removed from the female stimulus set to leave 30 images (six females with 2-7 images for each). In total, 60 colour images were tested.

To normalise the colour images, chromaticity values of the images were converted to CIE colour space values. The colour space values were then converted to red, green,

blue (RGB) values using the pre-defined matrix. Desired RGB values on the CRT screen were obtained by gamma correction of the monitor (Dobkins et al., 2000). This ensured that images were presented in device independent Yxy chromaticity coordinates. The image backgrounds were removed and replaced by a homogenous grey with a luminance matching the mean luminance of the image pixels. The mean image luminance (CIE 1931 Y, 2 degrees observer) of each image was calculated as if it were displayed on the monitor. The mean luminance of each image was then taken away from that image, so that its mean luminance was 0, with the standard deviation being the mean luminance contrast. The mean luminance of the complete set of images was added to each image, such that each image had the same mean luminance. Finally, CIE Yxy values were converted back to RGB using the inverse of the transform created when calibrating the screen. If pixel values fell outside of a displayable range, they were returned to their original values, which allowed for complete images whilst only slightly altering the mean luminance. The final mean luminance was calculated and the images were converted to Microsoft Indexed 255 Colour Bitmaps with dimensions of 326 x 326 pixels. Control images were scrambled images that were created in the same way as the greyscale images (described in section 5.2.2; Figure 12b).

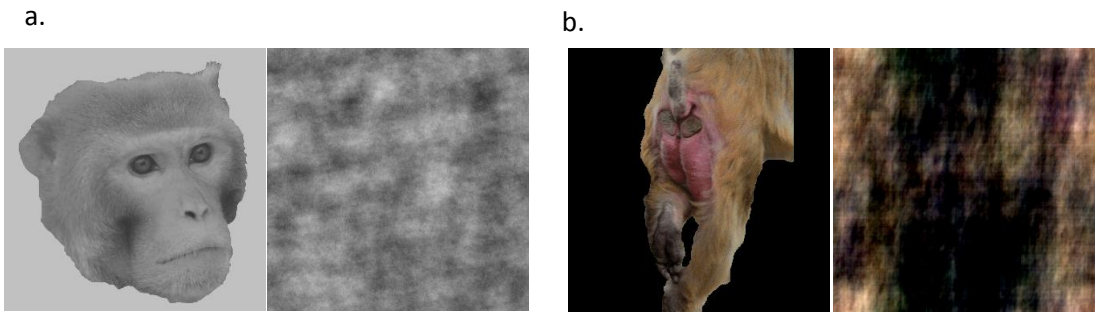


Figure 12. Examples of (a) greyscale and (b) colour social images and their matching control images (not to scale) which were 326 x 326 pixels and presented at 8.85 x 8.85 degrees of visual angle (dva).

5.2.4 Image Preference Test

Monkeys 1 and 2 were presented with greyscale images and Monkeys 3 and 4 with colour images to assess whether one type of image was more motivating than the other. Each monkey had to fixate on a central spot (0.5 x 0.5 dva; 5 x 5 dva eye window allowance) for 2000 ms, after which a juice reward of Ribena was given (~0.1 – 0.2 ml, dependent on the monkey). Following the initial 2000 ms of fixation, two images (8.85 x 8.85 dva) were presented for 5000 ms (Figure 13), during which the monkey was free to look wherever he chose on, or off, the screen. The monkey's right eye was tracked throughout these 5000 ms to ascertain which image he spent more time looking at (if any). Trials were conducted in blocks; once an image had been used, it would not be shown again until all other images had been presented. Once all images had been used, a new block began. This ensured that each image was shown an equal number of times per session. The images were shown in three different pairings, as follows: male vs scrambled control, female vs scrambled control, and male vs female. The control images were shown alongside their matching original monkey image, whereas male-female image presentations were paired at random. Reward was given after successful fixation, instead of at the end of a trial (following the 5000 ms image presentation), to ensure that no association could be made between the monkey's choice of which image to view and the fluid reinforcement.

This protocol was carried out for 4 days for each monkey. Following this, the eye tracking data were analysed and the image type (male face, female perinea or scrambled control) with the longest-associated average viewing time was taken as the monkey's preference. The preferred image type was specific to each animal, and animals received their own preferred image type in the fluid + image reward task (section 5.2.5).

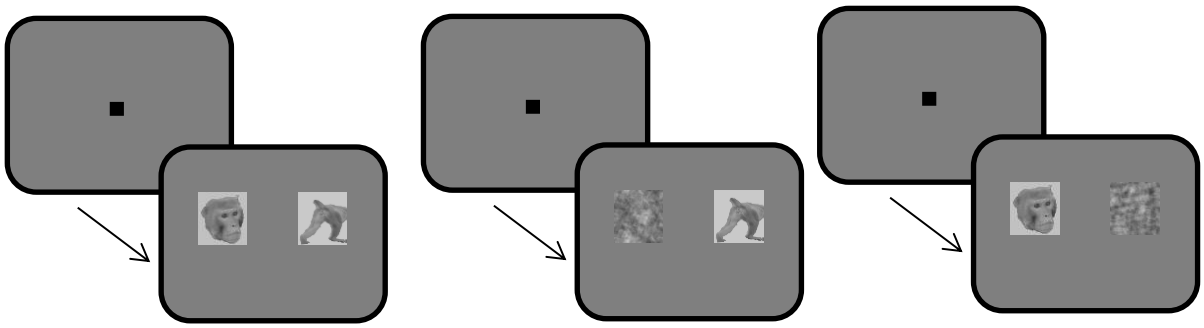


Figure 13. Social reward preference test (images not to scale). The monkey fixated on a central dot for 2 s before a pair of images was presented. The pairs could be either: (a) male face vs female perinea; (b) female perinea vs a matched control image; (c) or a male face vs a matched control image. Image pairs were presented for 5000 ms and the monkey could look wherever he chose for this duration.

5.2.5 Image Rewards

Two slightly different tasks were used to test if preferred images were rewarding in cognitive tasks, depending on each monkey's previous training. These are referred to as the fluid + image reward tasks. Monkeys 1 and 2 were required to hold a touch bar whilst fixating on a central square (0.5 x 0.5 dva; 5 x 5 dva eye window allowance) on the screen for 1650-2000 ms and release the bar within 1000 ms when the square dimmed (reduced in contrast). Monkeys 3 and 4 were required to fixate on a central square for 1000 ms with no bar release required. On completion of a correct bar release or fixation, the monkeys were confronted with one of three possible conditions (Figure 14). Either a single cross (condition 1) or a single annulus (condition 2) were presented randomly at either (x = -6, y = 6 dva) or (x = 6, y = 6 dva), or both stimuli were presented simultaneously (condition 3), whereby the location of the cross/annulus was assigned randomly to the two possible stimulus locations. To obtain a fluid reward (Ribena, ~0.1 ml) and the presentation of a non-preferred image stimulus (8.85 x 8.85 dva) for 2000 ms, the monkey had to make a saccade to the cross and fixate on it for 500 ms. To view an image from his preferred stimulus set (8.85 x 8.85 dva) for 2000 ms with no fluid reward, the monkey had to saccade to the annulus and fixate for 500 ms. If no saccade was made, a 2000 ms delay occurred before the next trial, without any reward (fluid or image) being given. Conditions 1 and 2 served as 'learning trials' to help the monkeys to establish the outcome of a saccade to each target. The choice trials (condition 3) established which the monkeys' preferred reward (fluid or image) was. Monkeys 1 and 2 had some prior training on the meaning of the saccade targets and so 20% of their

daily trials were learning trials and 80% were choice trials. Monkeys 3 and 4 were not trained beforehand on the meaning of the targets (due to time constraints) and so had an equal proportion of all three trial conditions daily (33% each), to ensure that they learnt what the targets represented.

To measure the number of trials performed solely for fluid, monkeys performed a control task (hereafter referred to as the fluid only task). This consisted of the annulus saccade target and a resulting fluid reward with no image presentation, as well as a 2000 ms delay between trials to mimic the timings of the fluid + image reward task timings. The two tasks (the fluid + image reward task and the fluid only task) were carried out in an ABBABAAB order over 8 days, at the monkeys' normal fluid allowance levels, to establish a baseline interest in the images.

After the task had been performed at the normal fluid allowance, the minimum daily fluid allowance of the monkeys was increased by 100 ml to decrease motivation for fluid reward. This allowed an assessment of whether the social stimuli had any motivational value when the animals were potentially less motivated by fluids. The fluid + image reward task and the fluid only task were then carried out for a further 8 days in the same order (ABBABAAB).

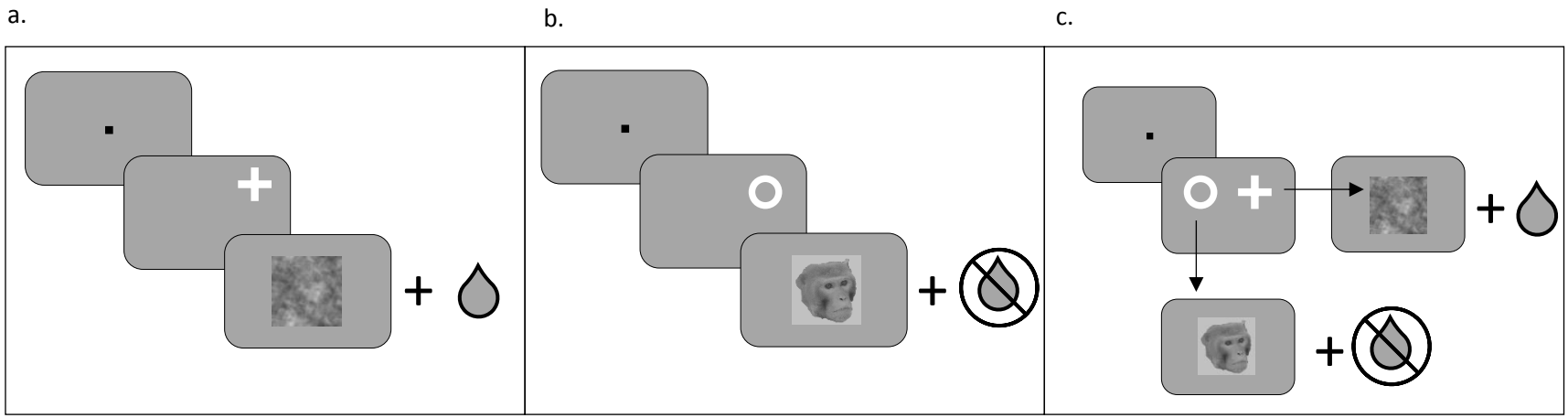


Figure 14. Fluid + Image Reward Task. After either a correct bar release (Monkeys 1 and 2) or fixation (Monkeys 3 and 4), the monkeys were confronted with one of three possible conditions: (a) a single cross (condition 1); (b) a single annulus (condition 2) or; (c) both a cross and an annulus were presented simultaneously (condition 3). Stimuli were presented randomly at either (x = -6, y = 6 dva) or (x = 6, y = 6 dva). To obtain a fluid reward and the presentation of a non-preferred image stimulus for 2000 ms, the monkey had to make a saccade to the cross and fixate on it for 500 ms. To view an image from his preferred stimulus set for 2000ms with no fluid reward, the monkey had to saccade to the annulus. If no saccade was made, a 2000 ms delay occurred before the next trial, without any reward (fluid or image) being given.

5.2.6 Video Rewards

Video reward assessments were made after the completion of the image reward assessments, when monkeys were still subject to an increased fluid allowance. The video reward task was designed to explore whether videos provided additional motivation for the monkeys already subject to an increased fluid allowance, and not to compare them directly to fluid-only rewards. It was not possible to normalise colour video, and therefore greyscale videos were presented to Monkeys 1 and 2 only. The monkey was required to hold a bar for 1650 - 2000 ms and release within 1000 ms when a central square dimmed in contrast, to gain a fluid reward. After completion of a correct bar release, he could then make an additional saccade to a cross saccade target to view a 2000 ms movie of a male or female conspecific (dependent upon his pre-established preference). The location of the cross was randomised to one of two positions (x = 6, y = 6 dva) or (x = -6, y = 6 dva). If no saccade was made, a 2000 ms delay occurred before the next trial. This was conducted for 5 days whilst the animal remained on the increased fluid allowance. The monkey's fluid allowance was then increased again to the average amount he would drink when given free access to water (Free Access Intake [FAI], as defined in Chapter 2, section 2.3), and the same task was carried out for a further 5 days.

5.3 Statistical Methods

All analyses were carried out using IBM Corp. SPSS (v21, SPSS Inc, Chicago, USA). Image preference was established by analysing the number of ms the monkey spent looking at each of the two presented stimuli per trial. These data were not normally distributed for any of the monkeys, could also not be transformed to normality and so were analysed using Wilcoxon Signed Rank tests.

The rewarding value of the social stimuli was investigated using *t* tests to compare the mean number of correct trials performed in the fluid + image reward task and the fluid-only task (the data were normally distributed and in cases where assumptions of equal variances were violated, *p*-values were adjusted as necessary). I also assessed preferences within the fluid + image reward task by calculating the percentage of times either fluid or social rewards were chosen in trials where the monkeys had a choice

(omitting the 'learning trials'). This allowed me to establish, in conditions when the monkeys were given a choice, whether they were motivated to work for fluid or image rewards.

To establish whether decreases in motivation to work for fluid rewards had occurred by increasing the daily fluid allowance by 100 ml, data from the fluid-only task were analysed. Data were normally distributed and *t* tests were used to determine differences in task performance between the normal and increased fluid allowances; I expected lower task performance once the fluid allowance was increased.

Video reward data were normally distributed and were analysed separately for each monkey at each fluid allowance level, by performing paired *t* tests to test for differences between choices for fluid rewards versus fluid rewards with an additional video presentation.

5.4 Results

5.4.1 Image Preference

All four monkeys significantly preferred one type of image more than the other two, however, they varied in their preferences (Figure 15) and there was no pattern relating to dominance rank. Monkeys 1 and 4 spent longer looking at the female perinea than the male faces (Monkey 1: W = 14.29; Monkey 4: W = 13.19, both $p < 0.001$) or the scrambled control images (Monkey 1: W = 18.03; Monkey 4: W = 15.76, both $p < 0.001$). In addition they viewed male faces for longer than scrambled controls (Monkey 1: W = 13.86; Monkey 4: W= 11.43, both $p < 0.001$). Monkey 2 preferred the male faces to the female perinea (W = 15.28, $p < 0.001$) and to the controls (W = 17.70, $p < 0.001$), moreover, he looked for longer at the female perinea than their scrambled controls (W = 8.70, $p < 0.001$). Surprisingly, Monkey 3 did not show a preference for either type of social stimulus, and instead looked at the scrambled control images for longer than the female perinea (W = 2.83, $p = 0.005$) and the male faces (W = 2.45, $p = 0.014$), and he had no preference between the male and female images (W = 0.04, $p > 0.05$). Therefore, in the fluid + image reward tasks, Monkeys 1 and 4 were shown images of female perinea, Monkey 2 was shown images of male faces, and Monkey 3 was shown images of scrambled control images.

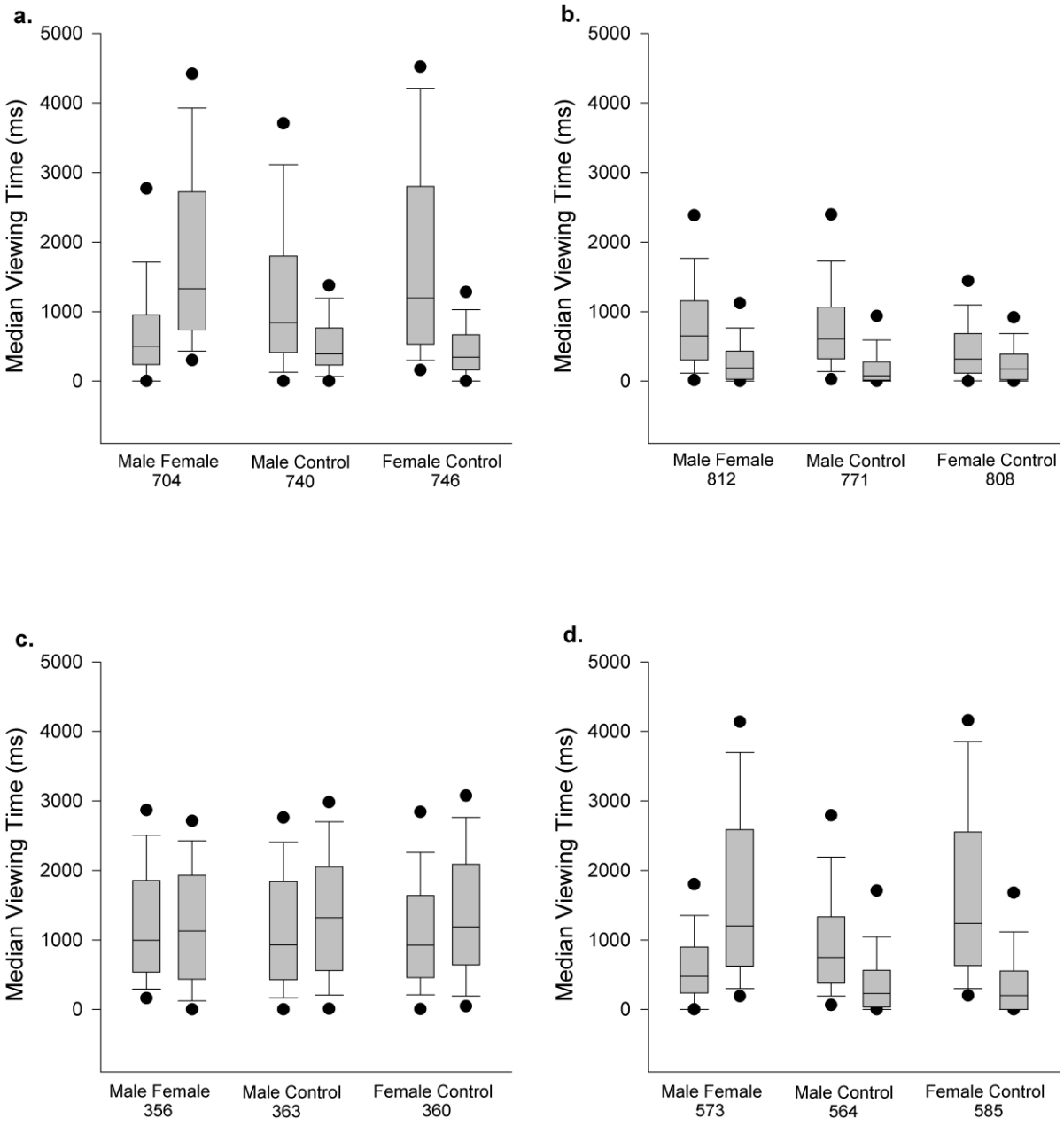


Figure 15. Median viewing times for pairs of images presented in the image preference test: (a) Monkey 1; (b) Monkey 2; (c) Monkey 3 and (d) Monkey 4. Closed circles represent the 5th and 95th percentiles. Numbers on the x-axis refer to the total number of each type of image pairing the monkey was presented with during the preference testing.

5.4.2 Using the Preferred Images as Rewards

At the normal fluid allowance, the average number of trials completed in the fluid + image reward task vs the fluid only task did not differ for three of the monkeys (Monkey 1: $t_{(6)} = 1.49$; Monkey 2: $t_{(4.8)} = 0.02$; Monkey 3: $t_{(6)} = 1.99$, all $p > 0.05$; Figure 16a-c). However, Monkey 4 (rewarded with colour images) performed more trials for the fluid + image reward task than for the fluid only task (Mean difference = 428, $t_{(6)} = 3.05$, $p = 0.022$; Figure 16d), suggesting that the inclusion of his preferred social stimuli increased his motivation to work. However, when assessing only the trials in which the monkeys had a choice between accessing a fluid or a preferred image reward, fluids were the favoured reward for all four monkeys, with all individuals choosing fluid rewards in over 98% of trials (Monkey 1 = 99.17% fluid choices, Monkeys 2 and 3 = 100%, Monkey 4 = 98.34%; Figure 16). These data show a strong preference for fluid rewards over preferred image rewards.

Given the strong preferences that all monkeys showed for fluid rewards at their normal fluid allowance, the daily fluid allowance was increased by 100 ml in an attempt to decrease motivation for fluids and assess whether this increased the motivational value of the preferred image rewards. Despite the increased fluid allowance and apparent reductions in fluid intake during the study, motivation in the fluid-only task was only significantly decreased for Monkey 2 when assessed individually (Table 10). During the increased fluid allowance, the average number of trials performed in the fluid only and the fluid + image reward tasks did not differ for any of the monkeys (Monkey 1: $t_{(6)} = 2.07$; Monkey 2: $t_{(6)} = 1.11$; Monkey 3: $t_{(6)} = 1.64$; Monkey 4: $t_{(6)}$; all $p > 0.05$; Figure 16), suggesting that working performance was still driven by fluids, and not by the image rewards (regardless of whether they were greyscale or colour). This finding is further strengthened by the fact that the increase in daily fluid allowance did not change the preference for fluid rewards, with all monkeys choosing fluid 100% of the time in choice trials.

Table 10. Difference in the number of trials performed for the fluid-only task at the normal fluid allowance and after fluid allowance had been raised by 100 ml and the associated *t* test values.

	Mean Difference	Standard Error of Difference	*t*-value	df	*p*-value
Monkey 1	-204.75	136.33	1.50	6	0.184
Monkey 2	-517.00	93.77	5.51	6	0.001
Monkey 3	-114.50	119.35	0.96	6	0.38
Monkey 4	-56.25	98.38	0.57	6	0.59

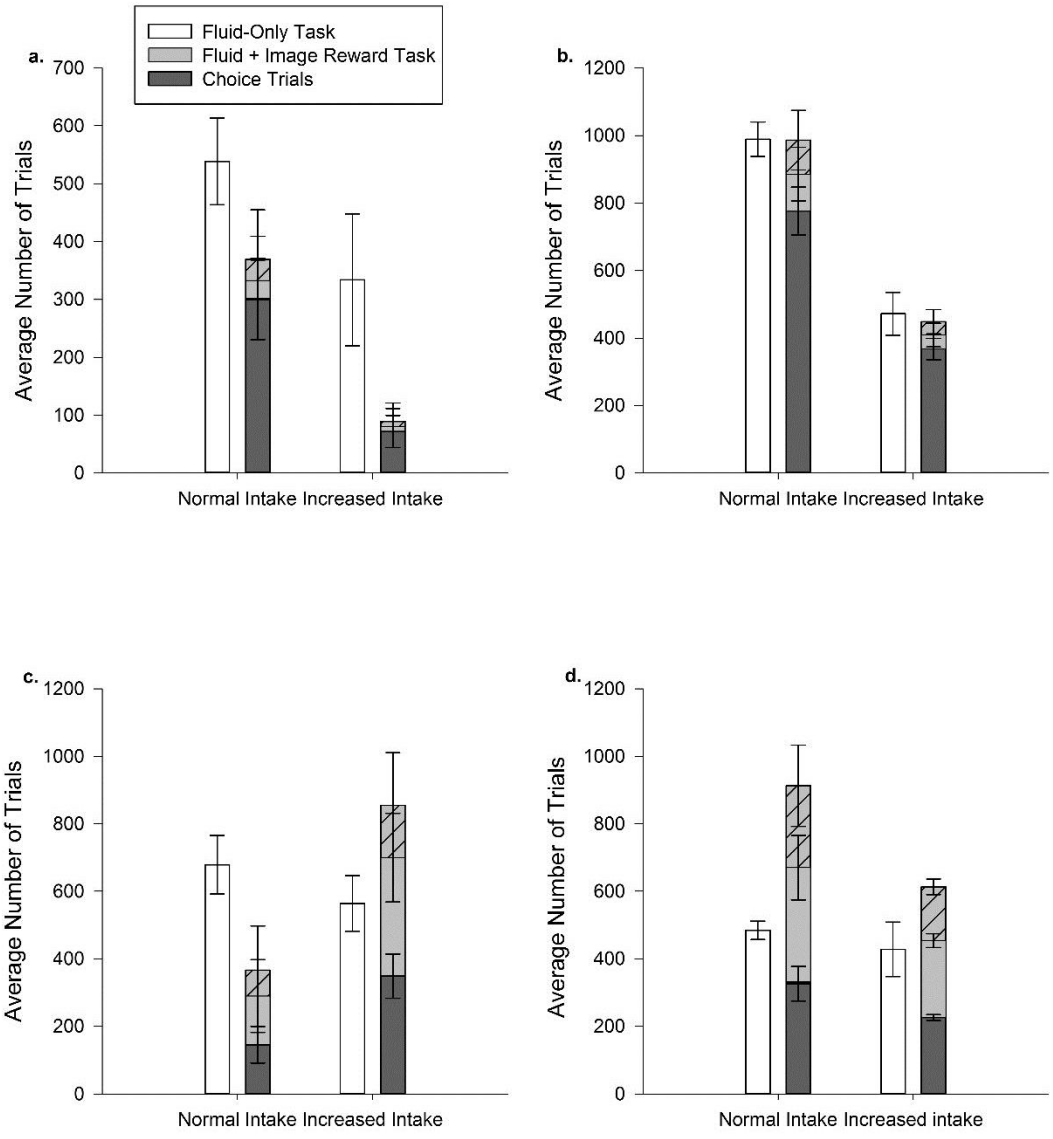


Figure 16. Average numbers of trials (±SEM) completed for the fluid only task (white bars), the image + fluid reward task including learning trials (all trials; all grey bars), and for the image + fluid reward task excluding the learning trials (choice trials only; dark grey portion of grey bars). Striped patterns represent choices for image rewards and solid fills represent fluid rewards. (a) Monkey 1; (b) Monkey 2; (c) Monkey 3 and (d) Monkey 4.

5.4.3 Video Rewards

Monkeys 1 and 2 remained on their increased fluid allowance whilst the efficacy of video rewards was assessed. The monkeys first received a fluid reward before having the option to make an additional saccade to view a video clip. The monkeys had the option to drink their initial fluid reward or to leave it. No fluid was ever observed under the drinking spout after testing and so the juice was assumed to have always been consumed. Both animals performed more trials for receipt of only fluids than for the fluid reward plus additional video reward (paired *t* test, Monkey 1: $t_{(4)} = 3.89$, $p = 0.018$; Monkey 2: $t_{(4)} = -5.41$, $p = 0.006$; Figure 17). However, both Monkey 1 and Monkey 2 both chose to view additional videos 26% and 31% of the time, respectively; indicating that they had some interest in the videos.

The daily fluid allowance was then raised to the monkeys' average consumption when given free access to water. The total number of trials performed per day decreased to an average 15.6 for Monkey 1 (13.6 for fluid only and 2 for additional video) and 17.2 for Monkey 2 (10.8 for fluid only and 6.4 for additional video). Monkey 2 continued to perform more trials for fluid only rewards than for the additional social reward ($t_{(4)} = 2.81$, $p = 0.048$; Figure 17). However, there was no difference in performance between the two reward conditions for Monkey 1 ($t_{(4)} = 0.80$, $p > 0.05$; Figure 17) These data suggest that although the videos were of interest to the monkeys, motivation still remained driven by fluid rewards.

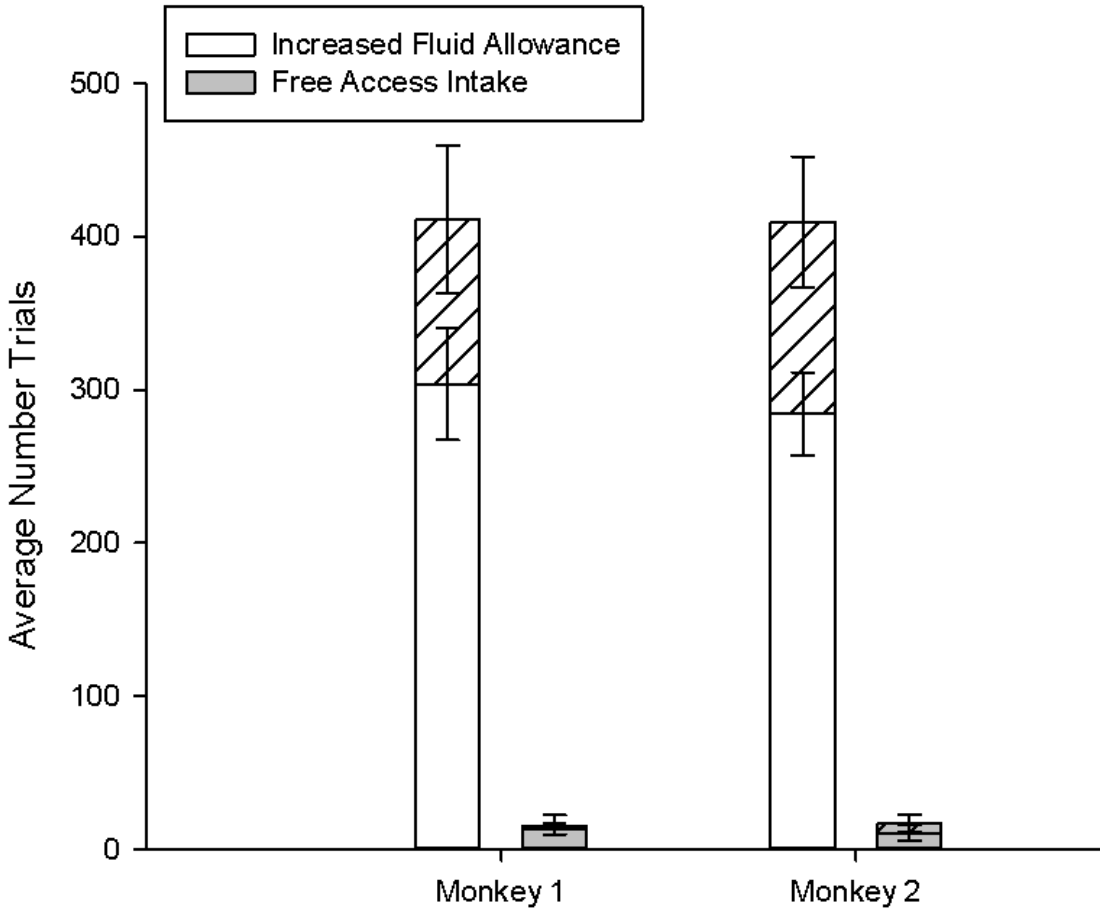


Figure 17. Average numbers of trials (± SEM) completed by Monkey 1 and Monkey 2 for fluid rewards and fluid rewards plus an additional video reward. Solid bars indicate fluid only rewards (no additional saccade for video reward). Striped bars indicate fluid rewards with additional video chosen.

5.5 Discussion

This study explored the social preferences of four laboratory rhesus macaques and the efficacy of using their preferred greyscale and colour stimuli as rewards for the successful completion of a single trial in a cognitive task. Despite earlier suggestions in the literature, I was unable to find evidence that social rewards could be used as an alternative (or supplement) to fluid rewards to motivate male macaques to participate for extended daily periods in a behavioural neuroscience task. These results have implications for refining current fluid restriction protocols for experimental animals.

Interestingly, the monkeys showed a wide range of preferences for the images shown to them. Whilst all four monkeys showed a strong preference for looking at one type of image, only three showed a preference for one of the social stimuli: two monkeys preferred to look at male faces, and one monkey favoured female perinea, as found in previous studies (Deaner et al., 2005; Klein et al., 2008). Surprisingly, one monkey preferred the scrambled control images over both female perinea and male faces. Speculatively, this could be because the control images were a novel stimuli, the type of which he had not been previously exposed to. Taken together, the data clearly show that it cannot be assumed that all monkeys will be interested in looking at social stimuli, and when they do, individuals may not prefer the same type of social stimulus (see also Ogura and Matsuzawa, 2012). This emphasises the need to ensure that if social stimuli are to be used as motivators in tasks, they need to be tailored to each individual prior to the experiment.

Given the clear and strong preferences to view one type of image that all the monkeys showed, it was also surprising not to see strong effects on animals' motivation to access the images through performance in the tasks. At their normal fluid allowances, only one of the monkeys (Monkey 4) performed more trials for the fluid + image reward task than for the fluid-only task. The remaining three monkeys did not differ in performance between the two tasks. Although these data suggest that the use of images could be rewarding for some animals, and increase their task performance, it was evident that when given a choice between social and fluid rewards, fluids were favoured and chosen over images in more than 98% of the trials for each monkey. Therefore, even for Monkey 4, where the number of trials performed had increased with

the inclusion of images, this was not driven by a motivation to voluntarily access social rewards, since they were rarely chosen. Consequently, there is no evidence that an animal's motivation to work was driven for the desire to access social (or preferred) stimuli.

When the daily fluid allowance of the monkeys was increased in an attempt to decrease motivation for fluid rewards, the monkeys continued to show a strong preference for fluids over images when given the choice. This could be because the increase in daily fluid allowance was not sufficient to reduce motivation for fluid rewards. Consistent with this idea, a reduction in daily performance in the fluid-only task occurred for only one of the animals; however, even for this animal, there was no increase in viewing of social rewards with the increased daily allowance. The allowance could have been further increased for the three other animals, however, this would have unnecessarily increased the study length. This risked the monkeys becoming more familiar with the images, making the data difficult to interpret as changes in the monkeys' motivation could have been confounded by habituation to the images.

Greyscale and colour images were not treated differently by the animals: individuals showed the same strong (individual) preferences for both types of social images. It was expected that the colour images would be more rewarding than the greyscale due the social salience of the red colouration However, Waitt et al. (2003) and Higham et al. (2010) suggest that the signals may be more valuable to female macaques and in this study at least, there was no evidence that red coloration produced a stronger preference or was more rewarding for the animals (see also Deaner et al., 2005).

Video rewards were investigated at the increased fluid allowance and when monkeys were given their average free access intake. The monkeys were rewarded with fluids and then had the option to view a short video clip of their preferred social stimulus. The videos proved to be of interest to the two monkeys to which they were shown, with both monkeys choosing to view the optional videos after they had received fluid rewards (Monkey 1: 26% of the time and Monkey 2: 31% of the time). This was the case even though the male monkeys were unfamiliar and the female monkeys were housed in the same colony. These results are in line with other studies which have demonstrated the efficacy of video rewards (Andrews and Rosenblum, 1993; Brannon

et al., 2004; Ogura and Matsuzawa, 2012). However, the monkeys were not as motivated to view the videos when given their free access intake. At both restriction levels (increased allowance and free access intake) monkeys could choose not to consume any fluid if they wished, and to still make the saccade to view the video. This was not observed to occur in either situation, perhaps indicating that the key driver in performance remained as fluids throughout this study.

Although there was some indication of motivation to view the images and videos, the majority of the findings presented here are not in line with previous studies which have successfully reinforced monkeys with social rewards (Andrews and Rosenblum, 2001, 2002; Deaner et al., 2005; Klein et al., 2008). This could be due to the monkeys' individual preferences for nutritive rewards instead of social rewards. This type of individual preference has been demonstrated previously in both rhesus macaques (Washburn and Hopkins, 1994; Washburn et al., 1997) and bonnet macaques (Andrews and Rosenblum, 1993; 2001), with some animals having been shown to favour video rewards of conspecifics, others preferring to receive a food pellet reward, and some showing no definite preference for either. Therefore, although the monkeys in this study show preference for nutritive fluid rewards over social rewards, I cannot be certain that social rewards would not be rewarding in a different population of laboratory animals, but the consistency of the four animals in this study in their choices for fluid would argue against that.

One possible reason for why a preference for a certain stimulus did not translate into motivating animals to perform a task is that the animals were socially housed. Every monkey was pair-housed, and had visual, auditory and olfactory contact with approximately 40 other rhesus macaques (both male and female) housed in the primate unit. Although some studies have successfully implemented social rewards with pair- and group-housed macaques (e.g Deaner et al., 2005; Klein et al., 2008), it may be that the level of social enrichment experienced on a daily basis by the monkeys in the study was too high for the images to be adequately valued as reward. It could be that images presented in cognitive tasks may be more rewarding to macaques that are socially restricted or deprived, such as those that are singly housed.

In this study, free-viewing image preferences established after the receipt of a valuable reward (fluid), did not translate into motivating rewards when paired against the choice of fluid rewards. This was potentially influenced by the many hundreds of times the images were presented in the preference testing stage of the experiment. This initial exposure to the images may have been sufficient for the monkeys to become habituated to, and uninterested by the stimuli; although previous studies have found evidence of prolonged exposure without habituation (Andrews and Rosenblum, 1993).

Finally, it is perhaps most likely that both the greyscale and colour stimuli are not sufficiently motivating on a trial-by-trial basis when many iterations of a task must be performed. Perhaps, instead of using the images trial-by-trial, the stimuli would be more useful as a "jackpot" reward (Westlund, 2012), occurring every 50 to 100 trials in addition to the fluid rewards, in order to enhance motivation, without risking an overuse the images. Alternatively, the stimuli could be used alongside smaller fluid rewards, as described by Deaner et al (2005) in order to lessen the fluid restriction. A larger stimulus set containing more individuals may evoke a maintained interest in the images and changing the stimulus set has also been shown as an effective way to increase interest in social rewards (Andrews and Rosenblum, 2001). However, the time and resources required to build a stimulus set such as that used in this study are not trivial. Moreover, implementing preferred stimuli presentation as rewards in an experimental setting where neuroscientific data are obtained is equally non trivial, as reward choices (preferred image vs. fluid) at the end of a trial increases the effort and trial time, which may thus offset the added motivation.

5.6 Conclusions

In conclusion, I found that preferred social stimuli, whether still images or videos, greyscale or colour, were not sufficient for motivating rhesus macaques to perform trials in cognitive tasks that are regularly used in behavioural neuroscience. Based on these data, social rewards cannot be recommended or discouraged as a viable strategy to refine of fluid restriction protocols, although future studies that build on these findings may find alternative reward schedules that could theoretically overcome the currently encountered limitations.

Chapter 6: Discussion and Conclusions

The aims of this thesis were to determine the impacts of fluid restriction protocols on rhesus macaques used in behavioural neuroscience and to attempt to refine these protocols through the use of preferred fluid rewards and social rewards. The studies have contributed much-needed data to fill gaps in the knowledge surrounding fluid restriction and clarify their effects on physiology, behaviour and scientific output. Providing data on the impact of techniques used in animal research helps both academics and the public to develop informed opinions on the suitability of their use.

6.1 The Controversy of NHP Research

Over recent years, details on how animals are used in science has become more accessible through agreements such as the Basel declaration (http://www.basel-declaration.org/), the Concordat on Openness on Animal Research (http://www.understandinganimalresearch.org.uk/policy/concordat-openness-animal-research/) and through organisations such as Understanding Animal Research (http://www.understandinganimalresearch.org.uk/). However, *in vivo* studies remain a highly debated topic and the general public's perception of animal research can be negative, with the research often seen as somewhat unnecessary (Leaman et al., 2014). There is a heightened sensitivity towards primate work in particular (Goodman and Check, 2002), most likely due to the phylogenetic and morphological closeness of NHPs to humans, as well as the fact that NHPs are often seen as more sentient than other commonly used laboratory animals, such as rats (see Broom (2014) for a comprehensive overview of sentience and animal welfare). To promote a greater understanding and acceptance of *in vivo* studies, the public need to be advised of the relevant legalities, current practices and animal welfare standards, and additionally about the real, not purported, implications on animal welfare. In line with this, the Weatherall Working Group Report on the Use of Non-Human Primate Research dedicated two of its 16 recommendations to the advancement of public engagement in NHP research. The report called for more frequent meetings between the media and scientists involved in primate studies, in order that accurate and up-to-date information is reported to the public (Weatherall et al., 2006).

As well as the need to be open about primate research, it is important that the methods required for effective NHP study are fully evaluated for both ethical and scientific reasons, to ensure that the protocols minimise harm, and that they do not result in unreliable data. The first part of this thesis made such an evaluation for two commonly used fluid restriction protocols.

6.2 The Impacts of Fluid Control

Past research has assessed singular consequences of fluid restriction such as behaviour (Hage et al., 2014) and blood physiology (Yamada et al., 2010), but the data presented here are the first to be collected using a suite of measures to evaluate the overall effect of fluid restriction protocols on macaque welfare and scientific output. Despite the widely held concerns that fluid restriction protocols impact negatively on NHPs (e.g. Orlans, 1991; Willems, 2009; Westlund, 2012), the experiments in Chapter 3 failed to detect any physiological harm or weight loss of any significance following two commonly-implemented protocols. Macaques are limited in their fluid intake in the wild, due to factors such as predation risk, intra- and inter-specific competition at watering sites (Lindburg, 1977) and seasonal rainfall variation (Lindburg, 1977; Malik, 1986). For example, during the winter, macaques in certain parts of Northern India obtain the majority of their water requirements from the abundance of fruit and vegetation in their environment. However, in the dry, summer season, when succulent vegetation is not as readily available, the monkeys must increase their daily time spent locating water from 2% to 4.7% (Malik, 1986). Given that macaques are exposed to a fluctuating availability of water in their natural habitats, it is perhaps not surprising that their bodies can cope with a reduced volume of fluid in a captive setting. Even so, it is an encouraging result of this thesis to find that 5-day and 7-day fluid restriction protocols are not physiologically harmful to macaques, easing concerns about their widespread implementation.

6.3 Refinement of Fluid Restriction Protocols

In addition to investigating the impacts of protocols used in *in vivo* research, it is similarly important to refine them. Refinement was a concept first brought to light by Russell and Burch (1959) who defined it as: “any decrease in the incidence or severity of inhumane procedures applied to those animals which still have to be used”. Despite the

fact that fluid restriction did not impact negatively on the macaques' physiology in Chapter 3, this does not mean that efforts to improve the technique should be lessened. One important improvement would be to accurately measure and define what an individual animal requires in terms of water provision and to reliably record their free access intake without the confounds of being split from a cage mate or drinking due to "boredom"; aspects which may inflate a free access volume. Refinement efforts may be more effective when all aspects of the fluid restriction protocols are understood, including the data on which restriction levels are based. .

The two refinements explored in this thesis aimed to keep scientific data quality high whilst reducing the severity of the fluid restriction. The first refinement was the use of preferred fluids combined with different rewards schedules, previously highlighted as an area for research by the NC3Rs Working Group Report (Prescott et al., 2010). Mixed results were achieved for both the fluid preference assessments and for motivating the monkeys with different reward schedules. A factor largely contributing to this was the individuality of the monkeys; fluids and schedules that evoked enhanced motivation in one monkey would not necessarily translate to success in another.

Individual differences were also found when testing the second refinement of social rewards. Social stimuli have been successfully used as rewards and enrichments in several previous studies (Andrews and Rosenblum, 2002; Deaner et al., 2005; Klein et al., 2008; Ogura and Matsuzawa, 2012). For this reason, in Chapter 5, I attempted to motivate the monkeys using photographs and videos of conspecifics. I first established preferences for subsets of these social stimuli and then allowed the monkeys to choose between their preferred stimuli and fluids as rewards. Although the monkeys showed interest in, and displayed different preferences for, male, female and control stimuli, none of the preferred stimuli was successful at motivating the monkeys to perform in a cognitive task when compared with fluid rewards. Despite the lack of success of the experiments in Chapter 5, possible alternative uses of the social stimuli are discussed below (Section 6.4.3).

6.4 Recommendations for Practice

Conducting studies such as those presented here is important not only for our basic understanding of motivation in macaques, but also as a way of informing best practice

for behavioural neuroscience. Below, I highlight some recommendations for the use of fluid restriction protocols, as well as comments on the use of preferred fluids and different reward schedules and the potential uses of social rewards.

6.4.1 Implementing Fluid Restriction Protocols

Arguably the most important finding of this thesis is that fluid restriction protocols can be implemented safely for male rhesus macaques. Nevertheless, there are still points to consider before using these protocols. For example, the behavioural observations in Chapter 3 uncovered decreases in food consumption behaviours (foraging, eating and chewing) when macaques were subject to a stricter fluid restriction. For this reason, behaviours in the home cage should be observed regularly, to establish whether patterns of foraging and eating are altered during periods of fluid restriction. In addition, small amounts of weight loss occurred as a result of fluid restriction (though not to the detriment of the animals). However, as each monkey had an individualised fluid allowance, the weight loss reported in Chapter 3 is specific to the individuals used for the study and should not be assumed to be a universal reaction to fluid restriction. It is imperative that body mass is monitored on each working day for a fluid restricted NHP, as recommended by both the NC3Rs Working Group Report (Prescott et al., 2010) and the primate care guidelines (NC3Rs, 2006). Weighing the monkeys frequently is particularly important for a 7-day fluid restriction protocol, for which this thesis cannot provide longer-term results of body mass changes.

6.4.2 Preferred Rewards and Different Reward Schedules

Chapter 4 demonstrated that macaques vary in their individual fluid reward preferences and in their response to varying reward schedules. The data showed that it can be beneficial to establish reward preference, in order that high levels of motivation can be maintained whilst the severity of fluid restriction is relaxed. At the normal fluid intake allowance, one of the monkeys was motivated by a variable reward schedule and, to a certain extent, a choice schedule. It is possible that these schedules could also prove motivating for monkeys in other facilities, but before implementing these reward schedules, they should be assessed for their practicality of use (e.g. whether additional equipment is required). The time required to implement potentially minor

improvements in animal welfare, should also be taken into account. Some of these assessments can be time consuming, thus keeping animals for longer and reducing scientific output for possibly minimal welfare gains. Additionally, the trade-off should be assessed between the potential benefits of a choice schedule (Catania and Sagvolden, 1980) and whether the additional time taken per trial could impact on the overall scientific output. It is also important to note that the use of high sugar fluids (e.g. fresh fruit juices) as preferred rewards could impact on the monkeys' dental health and that these potential concerns should be discussed with a veterinary team.

6.4.3 Social Rewards

The macaques showed interest in the images in the initial stages of the social stimuli study. However, the stimuli were not subsequently effective as rewards on a trial-by-trial basis for tasks requiring many hundreds of iterations. Therefore, it may be worth attempting to use these types of rewards in studies which require relatively low numbers of trials from the animals, or when an animal's housing situation requires them to be socially deprived. In these situations, the stimuli may prove to be more motivating, with the potential for lower rates of habituation. Furthermore, it may be possible to implement social stimuli as jackpot rewards, in which the monkey has access to photos or videos after completing, for example, 50 or 100 trials. Jackpots, in the form of fluid rewards, have been advocated as potentially rewarding for animals with the possibility of the jackpot serving to enhance motivation (Westlund, 2012), but there is currently no evidence they prove motivating in macaques performing cognitive tasks during electrophysiological or brain imaging studies.

6.5 Limitations of the Studies

I acknowledge that there are certain limitations to the studies in this thesis, caused by time constraints or by the nature of the experimental design, and I describe and explain these below.

It was not logical, nor ethical, to acquire NHPs to use specifically for the experiments in this thesis, and much of the work was carried out using animals undergoing breaks from neuroscience research. This presented two main problems. Firstly, it is time consuming and labour intensive to train a monkey to perform a certain task, but this is

somewhat worsened if the monkey is familiar with working with a different researcher. This can present barriers such as increased training times. Secondly, opportunistically using other researchers' animals results in small sample sizes, which is a main limitation with many NHP studies. However, I feel these limitations are acceptable for the ethical benefits they provide by decreasing the numbers of animals used in experiments and fulfilling another of the 3Rs, reduction (Russell and Burch, 1959).

Secondly, in Chapter 3, it was not possible to assess the subjective experiences of the monkeys undergoing fluid restriction; an element which would have been beneficial to be able to make inferences about states of thirst. Although there are protocols available to assess anxiety and cognitive state in NHPs, these were unsuitable for use in the study. For example, I attempted to implement a Human Intruder Test (Kalin and Shelton, 1989) to evaluate anxiety, but this led to harmful levels of aggression in the primate housing unit. Additionally, to conduct cognitive bias tests to detect underlying affective states (e.g. Harding et al., 2004) requires a great deal of control of potentially confounding variables. Had I measured cognitive bias whilst the monkeys were subject to different fluid restrictions, it would have been impossible, given the experimental design, to determine whether results were a direct consequence of the fluid restriction. They could equally have been attributable to the multitude of other variables impacting the monkeys' daily lives, such as social interactions, experience in the laboratory and hunger.

The third limitation relates to the social stimuli study. In Chapter 5, I discussed the possibility that a lack of motivation elicited by the social stimuli may have been because the stimulus set was too small and that using a larger number of photographs may have improved the study. However, both the greyscale and the colour photographs were subject to multiple, time consuming processes before they could be presented to the monkeys. This allowed for a controlled presentation of normalised images, allowing me to pinpoint any changes in motivation to the images themselves, rather than differences in luminance or hue intensity, for example. Although this resulted in a smaller stimulus set, I felt this was more beneficial than presenting a higher number of non-normalised images, which would potentially confound the results.

Despite the limitations of this thesis, the data presented contribute uniquely and meaningfully to the understanding of motivational techniques used for macaques in behavioural neuroscience.

6.6 Future Work

This project aimed to begin to understand the impact of fluid restriction on rhesus macaques and to explore possible refinements. Although the thesis provides a range of data, this area is still heavily understudied and much remains unknown. Areas for future research are given below:

- There are currently no published data pertaining to the long term effects of 7-day fluid restriction protocols. Since the monkeys at Newcastle were only subject to the 7-day restriction for the purpose of the experiments in Chapter 3, longer-term consequences (in the range of years) could not be drawn. As this protocol is used in other laboratories, it would be beneficial for others to explore long term effects and to establish the outcomes of the protocol. Given the data presented here, it would be useful if they simply performed a few blood sampling sessions in restricted animals, which will yield normative data to compare against existing data sets presented in this thesis.

- The psychological impacts of fluid restriction are not currently known. From the experiments in this thesis, it was not possible to infer the subjective experience of the animals; so although there was no physiological impact, I cannot be sure whether the monkeys experienced negative states as a result of thirst. One way to tackle this would be to use carefully designed cognitive bias and choice tasks to assess subjective states when the animals have free access to water versus when they are fluid restricted.

- As highlighted in Chapter 4, it would be beneficial to establish fluid preferences in a quick but accurate way. One way to achieve this would be to use a simple in-cage choice test when the monkeys are fluid restricted to the same extent as when working in the laboratory. However, this presents a trade-off between the monkeys being fluid restricted with no experimental

data collection for approximately a week, versus potentially gaining better quality data in the long term, with the possibility of less severe fluid restriction.

- As an alternative to fluid restriction, food restriction is also widely used (See Toth and Gardiner, 2000; Rowland, 2007) and equipment is available which allows for puréed foods to be delivered in much the same way as liquids (through a tube and into a mouthpiece). It would be interesting to assess whether monkeys could be given *ad libitum* access to water and rewarded with favoured puréed foods instead. These foods would supplement their daily diet of dried pellets and allow for free intake of water in the home cage.

6.7 Conclusions

The studies described in this thesis help to alleviate some of the concerns regarding fluid restricting NHPs in behavioural neuroscience, and offer potential refinements to fluid restriction protocols. Firstly, I show that two commonly used fluid restriction protocols caused no physiological harm to rhesus macaques, although further work is needed to determine long term effects of a 7-day protocol. Behaviour can be affected by both fluid restriction and by free access to water, and it is imperative that normal patterns of behaviour are established for monkeys, to be able to detect any changes resulting from imposed fluid restrictions. Secondly, when refining fluid restriction protocols, this thesis shows that each monkey should be treated as an individual and that preferred rewards, whether nutritive or non-nutritive, should be tailored to each animal. Continuing to evaluate and improve the protocols used in NHP research is beneficial not only to the reliability of the resulting science, but to creating a situation in which *in vivo* research is discussed in an open and progressive manner.

Appendix A

Supporting material relating to Chapter 3

Table 1. Paired *t* tests to assess whether the full length of the behavioural video recordings was needed or whether the middle hour of recording was sufficient. *t* tests were run analysing behavioural results from 07:00 – 09:00 versus 07:30 – 08:30 and 17:00 – 18:40 versus 17:20 – 18:20. Results are for each monkey, on each protocol for both morning and afternoon observations.

Monkey	Protocol	Time of Day	Mean	SEM	*t*-value	df	*p*-value
1	5-day	AM	.0000111	.0013876	-.008	17	.994
	7-day	AM	.0003399	.0019779	.172	21	.865
	5-day	PM	.0000024	.0026681	.001	16	.999
	7-day	PM	.0001331	.0040806	.033	15	.974
2	5-day	AM	.0000106	.0044411	.002	16	.998
	7-day	AM	.0000065	.0045833	.001	19	.999
	5-day	PM	.0000109	.0042730	.003	19	.998
	7-day	PM	.0000065	.0045833	.001	19	.999
3	5-day	AM	.0000040	.0036300	.001	14	.999
	7-day	AM	.0000024	.0050706	.000	16	1.000
	5-day	PM	.0000133	.0048236	-.003	14	.998
	7-day	PM	.0007253	.0040449	.179	14	.860
4	5-day	AM	.0003217	.0026259	.122	17	.904
	7-day	AM	.0013429	.0014821	.906	16	.378
	5-day	PM	.0000418	.0027072	-.015	16	.988
	7-day	PM	.0000088	.0037275	.002	15	.998

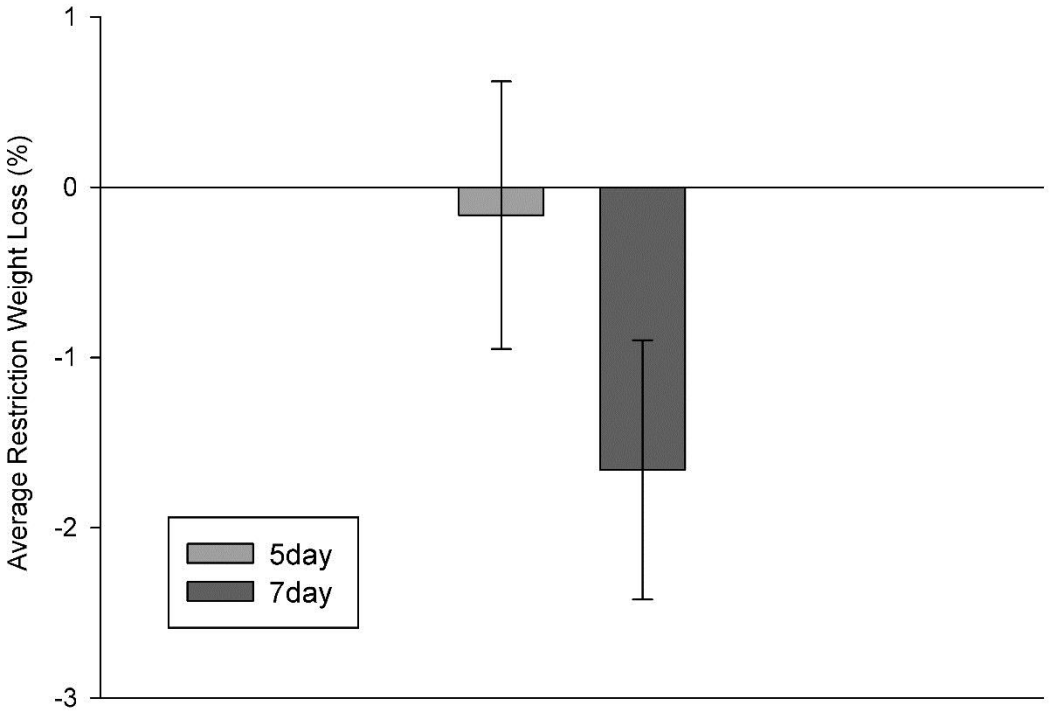


Figure 1. Average weight loss percentage for each four-week restriction block, averaged over all blocks for all monkeys (N = 4).

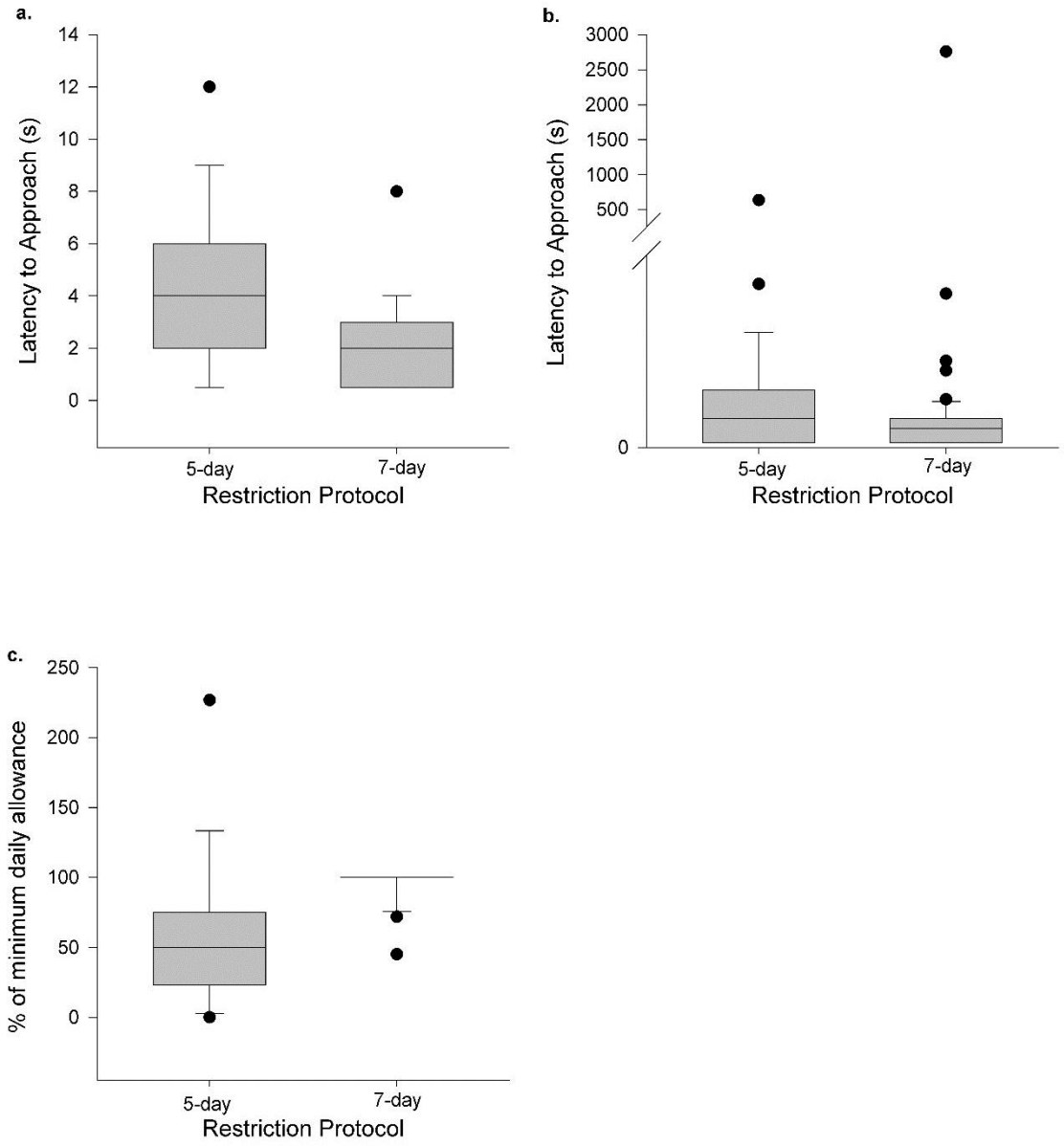


Figure 2. Median approach time to a bottle of water when attached to the cage on (a) Saturday morning and (b) Sunday morning. Note that y-axes have different scales. Filled circles represent outliers outside of the 10th and 90th percentiles. Water consumption after 5 min on Saturday (c), as a percentage of daily minimum allowances. Note, that more than 100% cannot be consumed on the 7-day regime.

Appendix B

Supporting material relating to Chapter 4.

Author	Species	Reward	Amount	Fluid Restriction	Food Restriction
Adachi and Hampton, 2011	*Macaca mulatta*	Food	-	-	-
Arsenault et al., 2014	*M. mulatta*	Juice	Experiment 1 & 2: 0.07 ml Experiment 2: 0.03 ml Experiment 3: 0.2 ml	-	-
Astrand et al., 2014	*M. mulatta*	Reward	-	-	Monkeys had free access to food pellets. They were also given fresh fruits and nuts.
Báez-Mendoza et al., 2016	*M. mulatta*	Blackcurrant juice, made from concentrate, diluted at a ratio of 1:11 by water (Ribena; GlaxoSmithKline, Middlesex, United Kingdom).	The number of cue circles (1–5) indicated the number of juice drops that the specific animal received; each circle predicted 0.2 ml of blackcurrant juice, delivered at 0.15s intervals	-	-
Ballesta and Duhamel, 2015	*M. fascicularis and M. mulatta*	Juice	Drop	*M. fasciularis*: water restriction with 1 day of free access to water each week. *M. mulatta*: To motivate the animals to perform the social decision task, and notably because of the presence of mildly aversive stimuli, access to water in	Animals were fed with monkey chow, fresh fruits, and vegetables

					the home cage was controlled. The animals normally earned between 50 and 200 ml juice during an experimental session. If the criterion of 25 ml/kg was not reached during a given session, extra fluid and fruits were given as needed at the end of each day to maintain proper fluid balance. Because the experiments were conducted over a period of several months, daily fluid intake was adjusted as needed to maintain an optimal motivation level corresponding to the monkey performing at least 100 correct trials per experimental session. No animal was let to reach a dehydration criterion (i.e., a loss of more than 10% of its weight)	
Baumann et al., 2015	*M. mulatta*	Juice Reward	-	-	-	

Beran and Smith, 2011	*M. mulatta*	Fruit flavoured pellets	94 mg	*Ad libitum* water.	Daily diet of fruits and vegetables independent of the amount of work they completed on the task.
Bethell et al., 2012	*M. mulatta*	Primate pellet	190 mg	*Ad libitum* water.	'20% protein, 5% fat, 10% fibre commercial dry primate diet (Diet 8773, Teklad NIB primate diet modified, Harlan Teklad, Madison, WI, USA) supplemented with fruit during morning and afternoon feeding rounds'
Blazquez and Yakusheva, 2015	*Do not state - 'macaque'*	Water	Every 1 - 1.5s	Standard water restriction protocols.	-
Bosking and Maunsell, 2011	*M. mulatta*	Juice	-	-	-
Braun et al., 2011	*M. mulatta*	Liquid	-	-	-
Burke et al., 2014	*M. radiata*	Fruit snacks, carrots, pears, gold raisins, grapes, or dried cranberries	One item	-	-
Canolty et al., 2012	*M. mulatta*	Liquid	-	-	-

Canteloup et al., 2015	*M. mulatta*	Raisin	One	Water was available *ad libitum* in the park	Feeding with commercial pellets took place in a one-acre wooded park. Fruit and vegetables were distributed twice a week in the park, outside experimental sessions.
Chang et al., 2011	*M. mulatta*	Cherry-flavoured juice	0.5 – 1.0 ml	-	-
Chang et al., 2012	*M. mulatta*	Cherry-flavoured juice	0.5 – 1.0 ml	At least 20 ml/kg of liquid daily in addition to fluid earned in the experiment. Usually earned 250 ml in testing (which fluctuated only by 50 ml across all sessions). Under *ad libitum* conditions, subjects drank approximately 500 ml	
Chao et al., 2015	*M. fuscata*	Food items	Given after every 100 stimuli	*Ad libitum*	The animal was given food (PS-A; Oriental Yeast Co., Ltd., Tokyo, Japan) *ad libitum* and also daily fruit/dry treats as a means of enrichment and novelty.
Chau et al., 2015	*M. mulatta*	Blackcurrant juice	Two 0.6 ml drops	Access to water 12–16 hr on testing days and with free water access on non-testing days	-

Chen and Stuphorn, 2015	*M. mulatta*	Water	1, 3, 5 to 9 units of water, where 1 unit equalled 30 ml	-	-
Chen et al., 2010	*M. mulatta*	Liquid	-	-	-
Chudasama et al., 2013	*M. mulatta*	Banana-flavoured pellets, half-peanuts, raisins, sweetened dried cranberries, “fruit snacks” or chocolate M&Ms.		Water was available *ad libitum*	All monkeys were fed a controlled diet of primate chow (catalogue number 5038, PMI Feeds Inc., St Louis, MO, United States of America) supplemented with fresh fruit or vegetables.
Cicmil et al., 2015	*M. mulatta*	Fluid	Reward size available for a correct choice on each trial depended upon the number of immediately preceding consecutive correct responses, increasing in two steps up to a maximum. 0.08 ml for one monkey on the first and second consecutive correct choices after an error, 0.12 ml for the third consecutive correct choice, and 0.2 ml on the fourth and all subsequent consecutive correct trials.	Animals worked on the task to gain fluid rewards to meet their daily requirements	-

			For monkey Fle, reward size was 1/3 of maximum for the first correct choice, 2/3 of maximum for the second, and reached maximum size (usually 0.18 ml) for the third and all subsequent consecutive correct choices.		
Curtis et al., 2015	*M. mulatta*	Grape	-	-	-
De Luna et al., 2014	*M. fascicularis*	45 mg purified dustless pellet (Banana flavor 5TUQ tab, Test-Diet, 1050 Progress Drive, Richmond IN 48384, USA), delivered via metal tube.	1	*Ad libitum*	Animals were maintained on a diet of fresh fruit, vegetables and monkey chow.

Deffains et al., 2011	*M. mulatta*	Liquid	0.3 ml	The monkeys were trained on weekdays and obtained their daily amount of liquid on these days during the testing sessions. Over the weekend, they had free access to water in their home cage.	-
Desrochers et al., 2015	*M. mulatta*	Juice or food slush	Reward was delivered for a constant duration across all trials: 0.2 s juice reward for monkey G and 0.25 s food slush reward for monkey Y	-	-
Dettmer et al., 2015	*M. mulatta*	Marshmallow or grape	1/2 marshmallow or 1/8 grape	-	-
Dunn and Colby, 2010	*M. mulatta*	Liquid	-	-	-
Falcone et al., 2013	*M. mulatta*	Apple, Water	Apple: Piece Water: 5 drops for Monkey P and 3 drops for Monkey C	The monkeys were on water restriction during the experiment receiving the water during the testing.	Primate food was available *ad libitum*. Additional fruits were given to the monkeys after the experimental session. On the weekend the water and fruits were given by the animal care takers once a day.
Falcone et al., 2012	Do not specify	Fluid	3 drops	-	-

Fernandez-Leon et al., 2015	*M. mulatta*	Grape (preferred) and cucumber (non-preferred)	Half a grape, quarter cucumber slice	-	-
Fiorillo, 2011	*M. mulatta*	Apple juice diluted to 2/3 of original strength	125µl or 50% chance of 250µl or 0µl	-	-
Fiorillo, Song, et al., 2013	*M. mulatta*	Juice, saline, and bitter solutions, Juice was two-thirds apple juice and one-third water. Saline was an aqueous solution of 8% NaCl (Monkey O) and 4% for Monkey F Bitter solution was 1 or 10 mM denatonium	Juice: 180µl of juice delivered over a period of 200 ms or 130 µl over 150 ms. Saline: 60 ms (30µl) in Monkey O, and 30 ms (10µl) in Monkey F Bitter: 80 ms (40µl), and was only tested in Monkey O. Whereas 1 mM was delivered during initial recordings of neurons, 10 mM was delivered during the latter recordings	-	-
Fiorillo, Yun, et al., 2013	*M. mulatta*	Juice, saline, and bitter solutions	The standard volume of juice delivered during neuronal recordings was 130µl, and it flowed for 150 ms. A larger volume of 240µl was used in experiments with juice omission. Saline and bitter	Liquid intake was restricted to ensure motivation to participate in experiments.	-

			solutions were 10–40 µl delivered over 30–80 ms.		
Fitzgerald et al., 2011	*M. mulatta*	Juice	-	-	-
Friedman and Selemon, 2010	*M. mulatta*	Food tailored to animal, unspecified	-	-	-
Fujimichi et al., 2010	*M. fuscata*	Juice	-	-	-
Ganguly et al., 2011	*M. mulatta*	Liquid	-	-	-
Ghose and Maunsell, 2012	*M. mulatta*	Juice	Drop	-	-

Glavis-Bloom et al., 2013	*M. mulatta*	a) plain, blue M&M candy, (b) unsalted peanut, (c) raw carrot, (d) raisin, (e) banana-flavoured pellet (1 g size), (f) raw radish, (g) garlic clove, (h) dog chow, (i) Altoid (curiously strong breath mints), and (g) Cheerio (bland oat cereal).	Seven food items selected from the food preference task for each monkey (the four most preferred and the three least preferred) were used to bait each of seven boxes	Water was available *ad libitum*	Maintained on a diet of monkey chow supplemented with fresh fruits and vegetables.
Golub et al., 2015	*M. mulatta*	Water	120µl for monkey A,120–130 µl for monkey C	-	-
Gremmler et al., 2014	*M. fascicularis*	Water	defined amount	-	-
Gu and Corneil, 2014	*M. mulatta and M. fascicularis*	Liquid	-	-	-
Haley et al., 2011	*M. mulatta*	Preferred candy	-	-	-
Hanks et al., 2014	*M. mulatta*	Liquid reward	-	-	-
Hayden et al., 2010	*M. mulatta*	Juice	67, 200 or 333µl	Monkeys were placed on controlled access to fluid outside of experimental sessions.	

Hayden et al., 2011	*M. mulatta*	Juice	67, 200 or 333µl	Monkeys were placed on controlled access to fluid outside of Experimental sessions.	-
Heimbauer et al., 2012	*M. mulatta*	Food pellet	One 97 mg pellet. One monkey given two pellets for motivation reasons.	No restriction.	No restriction.
Heiney and Blazquez, 2011	*M. mulatta*	Reward	-	Water restriction	
Hunt et al., 2015	*M. mulatta*	Fruit Juice	Trial type: 0.26, 0.40, 0.55, 0.65ml,Trial Type: 0.36, 0.56, 0.77, 0.90 ml.	-	-
Isbaine et al., 2015	*M. mulatta*	Fruit juice	Drops.	Their liquid consumption and their weight were carefully monitored on a daily basis early in their training, and on a weekly basis during the steady phase of the experiment.	The two monkeys were maintained on a dry diet for the duration of the study.
Jacob and Duffy, 2015	*M. mulatta*	Liquid	-	-	-
Jang et al., 2015	*M. mulatta*	Food	-	-	-

Jedema et al., 2010	*M. mulatta*	Water	Task 1: 0.016 ml/kg or 0.08 ml/kg Task 2: 0.07 ml/kg or 0.016 ml/kg Task 3: 0.07ml/kg or 0.014 ml/kg Task 4: 0.075 ml/kg Task 5: 0.15ml/kg or 0.03 ml/kg.	Monday-Friday, animals received 25ml/kg/day of water, Saturday and Sunday: *Ad libitum* water.	Animals were fed sufficient monkey chow biscuits (Purina) to maintain healthy body weight plus fruit treats daily.
Jiang et al., 2015	*M. mulatta and M. radiata*	Juice	0.1-1 ml drop. Correct choices were encouraged by progressively increasing the reward size for consecutive correct trials.	-	-
Jones et al., 2010	*M. mulatta*	Water	1.16 ml	Both monkeys were kept on a water-restricted diet approved by an institutional animal care and use committee.	
Konoike et al., 2012	*M. fuscata*	Fruit Juice	Drop	-	-
Koval et al., 2011	*M. mulatta*	Juice	-	-	-
Kralik, 2012	*M. mulatta*	Banana flavoured pellets	45 mg Different amounts of pellets were given for different trials.	-	-
Kunimatsu et al., 2015	*M. fuscata*	Liquid	-	Water intake of monkeys was controlled on a daily	-

				basis so that they were motivated to perform the tasks.	
Lanz et al., 2013	*M. fascicularis*	Banana-flavoured pellet	One	Free access to water	When a 10% loss of weight was measured, experiments were interrupted until they recovered their previous weight. Such event did not occur in the course of the present study. They were never deprived of food but the daily intake was adjusted to the performance in order to not loose motivation.
Lee et al., 2015	*M. mulatta*	UK animal received food pellet rewards whereas the U.S. animals received juice reward	-	-	-
Liu et al., 2010	*M. fascicularis and M. mulatta*	Reward	-	-	-
Livingstone et al., 2014	*M. mulatta*	Liquid	0-25 drops, corresponding to the magnitude represented on whichever side of the screen he touched.	They were allowed to work to satiety each day, usually performing > 500 trials per day.	Each monkey spent 2–4 h per day alone, with food, in the training cage, 7 days per week.

Livingstone et al., 2010	*M. mulatta*	Juice or water	Rewarded by the same number of drops as the numerosity of the chosen stimulus/	For the first 3 months of training, they were given *ad lib* water during non-test periods, and juice rewards. After they stopped taking the free water in their cages and were drinking predominantly the juice provided during test periods, we switched the reward to water and stopped providing fluids before the daily testing period. We offered water in the afternoon, after testing, but the monkeys usually did not take any; their daily fluid intake was always more than 30 ml/kg.	They always had *ad lib* food and have been steadily gaining weight.
Mandell et al., 2011	*M. nemestrina*	Fruit	Small piece.	-	-
Mante et al., 2013	*M. mulatta*	Reward	-	-	-
Marciniak et al., 2014	*M. mulatta*	Juice reward	-	-	-

Masse et al., 2012	*M. mulatta*	Reward	-	-	-
Matsumoto et al., 2016	*M. mulatta*	Liquid, Banana-flavoured pellet food reward	Task 1: 1 or 3 drops Task 2 and 3: 1 drop Task 4: one pellet.	-	-
Matsuo et al., 2011	*M. mulatta and M. fuscata*	Apple juice	Drop.	-	-
Meyers et al., 2012	*M. mulatta*	Fruit juice	-	-	-
Mitchell et al., 2014	*M. mulatta*	Fluid	-	All fluid restrictions in NHP were performed in accordance with the Salk Institute IACUC Policies. As such, all procedures were scientifically justified and approved in the IACUC protocol. Consideration was given to using positive reinforcement instead of restriction whenever possible. When necessary, the lowest level of restriction was used to achieve the scientific objective. Even though the macaques typically learn to meet their entire daily	-

			fluid requirement during a working session, a number of precautions were taken to avoid the possibility of acute or chronic dehydration or clinical disease due to fluid restriction. To this end, the attending veterinarian performed a full physical examination (including CBC, biochemistry and urine analysis) prior to enrolment in an approved study involving fluid restriction. Clearance for continued participation was renewed at each semi-annual physical examination. Sick animals or those on treatment were prohibited from being enrolled in fluid-restriction studies. While on restriction, each macaque received at least 20 ml of fluids/kg daily and was not fluid-restricted for

			more than 5 d each week. The laboratory and animal care staff monitored the animal's health daily and maintained accurate records on total daily food and fluid consumption (including treats in the laboratory). Abnormal behaviour, decreased food consumption, weight loss, or urine specific gravity exceeding 1.040 was reported immediately to the attending veterinarian for evaluation.	
Monfardini et al., 2014	*M. mulatta*	Chocolate candies	The animals had free access to water.	Received normal food rations of fresh fruits and monkey chow once a day after the testing session. On a daily basis, monkey chow and fruits were hidden in primate rubber toys, and bird seeds were scattered in the litter shavings so that the animals spend a good part of their day foraging.

Monosov et al., 2015	*M. mulatta*	Apple juice	Experiment 1, 2 and 5: 0.4ml Experiment 3: 0.4, 0.3, 0.2, 0.1 or 0 ml Experiment 4: 0.15, 0.2, 0.1, 0.25, 0.05 or 0.3 ml	-	-
Murray et al., 2015	*M. mulatta*	Rewards consisted of two of the following three foods: M&Ms, peanuts, and skittles	½ peanut, 1 m&m/skittle. Rewards were given in training and reward devaluation stage, not during the scan.	*Ad libitum* water in the home cage.	For the duration of the study, the monkeys were given controlled access to food to ensure sufficient motivation to respond in the test apparatus.
Mustafar et al., 2015	*M. fascicularis*	Pellet	No food reward was provided during the behavioural recordings. However, during the 2min inter-block interval, fifteen 45mg pellets were given to the monkeys regardless of their gaze behaviour.	Water available *ad libitum.*	They were maintained on a diet of fresh fruit, vegetables, and monkey chow.
Nejime et al., 2015	*M. fuscata and M. mulatta*	Water	Drop.	Water was withheld before each daily session, and was given as a reward in an experimental room. Supplemental water and vegetables were given after the session.	-

Nelissen and Vanduffel, 2011	*M. mulatta*	Fruit juice	-	-	-
Nelson et al., 2011	*M. mulatta*	Yoghurt or apple sauce	-	Access to water throughout experiment.	Access to food throughout experiment.
Nielsen et al., 2012	*M. mulatta*	Juice	-	-	-
Nienborg and Cumming, 2014	*M. mulatta*	Liquid	-	-	-
Noonan et al., 2010	*M. mulatta*	Noyes Sucrose Pellets	190 mg (one).	Had 24 hour *ad libitum* access to water, apart from when they were testing.	A large metal food box, situated to the left below the touch screen, contained each individual's daily food allowance (given in addition to the reward pellets), consisting of proprietary monkey food, fruit, peanuts, and seeds, delivered immediately after testing each day. This food was supplemented by a forage mix of seeds and grains given 6 h before testing in the home cage.
Ohyama et al., 2014	*M. mulatta*	Juice	Drop.	-	-

Padberg et al., 2010	*M. mulatta*	Primate chow-based flavoured pellets (Bio-Serv) and fresh or dried fruit or vegetable pieces	One item.	-	Each monkey's feeding schedule was monitored and adjusted throughout the training period under the recommendations of the veterinary staff to keep the animal motivated to work diligently and to maintain body weight within 10% of the original weight.
Parr, 2014	*M. mulatta*	Food reward, Diluted yoghurt	Small 9ml/min.	Not water restricted for participation in these studies.	Not food restricted for participation in these studies.
Paxton et al., 2010	*M. mulatta*	Banana flavoured pellets and chocolate candy	-	*Ad libitum* water.	Animals received a full ration of food daily.
Pearson et al., 2010	*M. mulatta*	Juice	150 – 400µl	Access to fluid was controlled outside of Experimental sessions; monkeys earned roughly 80% of total daily ration by performance.	
Rajalingham et al., 2015	*M. mulatta*	Juice	Small	-	-

Rudebeck and Murray, 2011	*M. mulatta*	M&Ms (Mars), Half peanuts, Raisins, Craisins (OceanSpray), Banana-flavoured pellets (Noyes), Fruit snacks (Giant Foods)	-	*Ad libitum* water.	-
Rudebeck et al., 2014	*M. mulatta*	Fluid, Peanut	Task 1: 0.5ml Task 2: 0ml Task 3: (3 x 0.1ml) or (1 x 0.5ml) Task 4:(3 x 0.1ml) Task 5: half peanut	Monkeys' access to water was controlled for 6 days a week.	Monkeys' access to food was controlled for 6 days a week.
Sadeghi et al., 2010	*M. mulatta*	Juice	-	-	-
Sadtler et al., 2014	*M. mulatta*	Juice	-	-	-
Sayers et al., 2015	*M. mulatta*	Food pellet	One	Water was continuously available during testing.	The monkey was not food deprived for testing.
Schmitt et al., 2014	*M. fascicularis*	Raisins and peanuts	-	Water was always available *ad libitum*.	They were not food deprived for testing. The monkeys were fed regular monkey chow, fruits and vegetables twice a day.

Schmitt et al., 2012	*M. fascicularis*	Raisins, peanuts, fruit	Between 1-8 pieces. Number of food reward pieces depended on the task carried out, and in certain cases on the choice of the animal (e.g. the subject pointed to the number of rewards it wished to receive).	*Ad libitum.*	No restriction.
Schneider et al., 2013	*M. mulatta*	Sugar pellet	One	*Ad libitum* water	Restricted.
Seif and Reza, 2015	*Do not report*	-	-	-	-
Sirotin and Das, 2010	Do not specify	Juice	-	-	-
Smith et al., 2013	*M. mulatta*	fruit-flavoured chow pellet	Versions 1- 4: 1 x 94mg pellet Version 5-8: 2 x 94-mg pellet	They had continuous access to water.	The animals were neither food deprived nor weight reduced for the purposes of testing.
Smith et al., 2015	*M. mulatta*	Fruit-flavoured primate pellets	-	They had continuous access to water.	They received a daily diet of fruits and vegetables independent of their efforts on the task, and thus they were not food deprived for the purposes of this experiment.

Snyder and Smith, 2015	*M. mulatta*	Liquid	-	-	-
Sripati and Olson, 2010	*M. mulatta*	Juice	Drop	-	-
Stoewer et al., 2010	*M. mulatta*	Juice	Drop	-	-
Sunkara et al., 2015	*M. mulatta*	Juice	Drop	-	-
Taffe, 2012	*M. mulatta*	Food pellet	One	*Ad libitum* water.	Daily chow (LabDietR 5038, PMI Nutrition International, Richmond, IN, USA; 3.22 kcal of metabolisable energy (ME) per gram) allocations were supplemented with fruit or vegetables 7 days per week.
Taubert et al., 2015	*M. mulatta*	Fluid	-	-	-
Van Le et al., 2013	*M. fuscata*	Juice	0.8 ml	The monkeys were deprived of water in their home cage. Supplemental water and vegetables were given after each day's session.	Food available *ad libitum*.
Voloh et al., 2015	Do not state - *'macaque'*	Liquid reward	High- and low-reward magnitude was 0.76 and 0.4 ml per successfully performed trial.	-	-

Walton et al., 2010	*M. mulatta*	Noyes sugar pellet	190 mg (unspecified).	*Ad libitum* water.	A large metal food box, situated to the left below the touch screen, contained each individual's daily food allowance (given in addition to the reward pellets) consisting of proprietary monkey food, fruit, peanuts and seeds, delivered immediately after testing each day. This was supplemented by a forage mix of seeds and grains given ~6 hours prior to testing in the home cage.
Wang and Dragoi, 2015	*M. mulatta*	Juice	5 drops.	-	-
Wilke et al., 2010	*M. mulatta*	Juice for saccade tasks and unspecified fruit for reaching tasks	Juice amount unspecified, 1-4 pieces of fruit.	-	-
Wright et al., 2013	*M. mulatta*	Fruit flavoured nonhuman primate tablets	Two 190-mg tablets.	Water was available *ad libitum.*	Each monkey was fed approximately 37 g of chow/kg bodyweight/d, and their diet was supplemented with fresh fruit and a multi-vitamin tablet (Kirkland Signature Sugar-free

					Children's Chewable Vitamins, Seattle, WA). Monkeys were fed approximately 20% of their daily chow at least 1 hour before the morning testing sessions. The balance of the daily food ration was provided after all the monkeys had finished working.
Wright Jr. et al., 2012	*M. mulatta*	Food pellet	One.	*Ad libitum* water	Daily chow allocations were supplemented with fruits or vegetables 7 days per week.
Yamada et al., 2011	*M. fuscata*	Water	0.032 ml／kg.	-	-
Yang et al., 2010	Do not specify	Liquid	-	-	-
Yanike and Ferrera, 2014	*M. mulatta*	Liquid reward	Safe outcome: 2 or 3 drops of water (0.1 ml each). Risky outcome: one of two sizes of reward, such that the average reward was the same between safe and risky trials.	-	-
Yoshida et al., 2011	*M. fuscata*	Isotonic water	-	-	-

Yoshida et al., 2012	*M. fuscata*	Isotonic water	-	-	-
Zhang et al., 2013	*M. mulatta*	Liquid	Large reward (0.2– 0.9ml of liquid), Aversive air puff toward the face, Little or no reward (either 0ml or <0.1 ml of liquid depending on task version).	-	-
Zhou et al., 2015	*M. fascicularis*	Apple	Piece.	They had free access to water.	They had free access to food. Briefly, monkeys did not have access to food for 4 to 6 h.

Appendix C

Data published from this thesis

Gray, H., Bertrand, H., Mindus, C., Flecknell, P., Rowe, C., Thiele, A., 2016. Physiological, Behavioral, and Scientific Impact of Different Fluid Control Protocols in the Rhesus Macaque (Macaca mulatta). eneuro 3.

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